

STUDIES ON THE INDUCTION OF CHITINASE BY ETHYLENE
IN ABSCISSION ZONES FROM BEAN (Phaseolus vulgaris).

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ABSTRACT

It is shown that bean abscission zones accumulate chitinase on treatment with ethylene. The presence of ethylene was found to be essential for the accumulation to occur. Chitinase was purified to homogeneity and used to raise antibodies in rabbits. By enzymatic assay and immunoassay, chitinase was found to be similarly present in bean stem, petiole and leaf. The time course of induction of chitinase followed similar kinetics in every tissue tested, but reached lower specific activities in the stem. The in vitro translation product of chitinase mRNA was found to have a mw of 32 kD. It was identified by immunoprecipitation, processing by microsomal membranes, affinity chromatography and the kinetics of accumulation. Addition of microsomal membranes to the in vitro translation system processed the 32 kD product to one of 30 kD, which is the mw of mature chitinase. The 32 kD product could be immunoprecipitated by the chitinase antiserum. An affinity substrate specific for chitinase was shown to be able to select the 32 kD from an in vitro translation mixture. The kinetics of accumulation on induction by ethylene ^{were} ~~was~~ similar for chitinase and the 32 kD product. Auxins had no effect on the accumulation of chitinase as detected by in vitro translation. Two cDNA libraries were prepared and screened for clones containing chitinase sequences. One clone was

identified, pABS 17, from the first library which by hybrid release contained sequences for chitinase. Further examination showed this clone contained in addition sequences for ribosomal RNA and was thus unsuitable as a probe for chitinase mRNA. Several clones were identified from a second library which contained sequences of ethylene regulated products. Hybrid release data on these was not unequivocal and their identity remains uncertain.

A partial amino acid sequence from the amino terminal end of chitinase was obtained. From this, an oligonucleotide sequence was prepared and used to screen a bean genomic library. However, no genomic clones containing chitinase sequences were identified.

ABBREVIATIONS

| | |
|--------------|--|
| ATP | Adenosine 5' triphosphate |
| cDNA | DNA copied from mRNA |
| cpm | Counts per minute |
| CTP | Cytosine 5' triphosphate |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| ds | Double stranded |
| EDTA | Ethylene diamine tetra acetic acid (disodium salt) |
| kD | Kilodalton |
| MOPS | Morpholinepropane sulphonic acid |
| mw | Relative molecular weight |
| OD | Optical density |
| pfu | Plaque forming units |
| PGS | Plant growth substances |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. |
| ss | single stranded |
| Tris | Tris (hydroxymethyl) amino methane |
| Triton X 100 | Octylphenoxypolyethoxy ethanol |
| (v/v) | Volume per volume (as a percentage) |
| (w/v) | Weight per volume (as a percentage) |

1.1 INTRODUCTORY REMARKS

The purpose of this chapter is to set the results described in the chapters 3-6 within the context of higher plant development and the modulation of gene expression by plant growth substances (PGS). To this end, the regulation of gene expression by PGS and the process of abscission, the model system used here, will be briefly reviewed with emphasis on the role of ethylene and its mode of action at the molecular level. As the main enzyme concerned in this study, chitinase, an ethylene-regulated enzyme, is believed to be involved in the defense of the plant against pathogens, the different mechanisms of defense that plants have against pathogens will also be briefly discussed.

1.2 PLANT DEVELOPMENT

It is generally accepted that development and differentiation involve the selective control of gene expression. Many cells in a differentiated plant remain totipotent but only a small proportion of the genome is expressed (Goldberg, 1983). This clearly indicates that plant gene expression is strikingly regulated. However, the exact way that this regulation is accomplished is still unknown. Modern approaches tend to explain plant development not only as a result of differential gene expression but also in terms of a complex series of interactions of both external and internal factors

(Bryant, 1976; Trewavas, 1976; Wareing, 1977; 1982). In this respect PGS, as integrating and coordinating agents are believed to play a key role.

1.3 PLANT GROWTH SUBSTANCES

1.3.1 General effects on plant development

A comprehensive review of PGS action is outside the scope of this thesis and it has been covered in a number of textbooks and reviews (Leopold, 1964; Wareing and Phillips, 1982; Trewavas, 1981). However, a brief account of their action in plants will be discussed. Ethylene, being the main PGS concerned in this study, will be treated separately.

There are five major PGS known in plants: auxins, gibberellins, cytokinins, ABA and ethylene. Research carried out over the last 50 years or so has shown that PGS are essential factors or adjuncts of many developmental stages. Processes such as cell division, cell elongation, abscission, flowering, seed germination, fruit ripening etc have all been related to PGS action in one way or another (Wareing and Phillips, 1982).

One striking feature of PGS is that their action is dependant on the particular tissue. For instance, gibberellins, will induce elongation of a developing barley leaf (Wareing and Phillips, 1982) whereas an aleurone cell of the same plant will respond by producing alpha-amylase (Yomo and Varner, 1971). Thus, the specificity of the response is built into the cell

by its previous developmental programme (Trewavas, 1976).

The action of PGS has been suggested to be mediated by receptors on the plasmalemma and elsewhere (Kende and Gardner, 1976; Venis, 1977). These receptors would, in turn, amplify the action of PGS and thereby bring about specific changes in plant metabolism. The assumption has been made that the receptors for PGS are proteins as it is believed that only proteins may be able to recognize subtle structural differences between small molecules (Kende and Gardner, 1976). These proposals are based largely on analogies drawn from the animal field in which several hormone receptors have been clearly identified (Jensen and DeSombre, 1972; O'Malley and Means, 1974). In the case of plants some binding proteins have been detected for auxins (Venis, 1977) and ethylene (Sisler, 1979; Bengochea et al . 1981), although it is still not known if they are receptors.

PGS have been shown to have an effect on membrane physiology. Each PGS may interact somehow with membranes as they all have hydrophobic properties. Auxins, gibberellins, cytokinins and ABA are molecules with a hydrophobic group attached to a single charged (and thus hydrophilic) moiety. Such molecules generally show greater solubility in organic solvents than in water and this property is also shared by ethylene. Thus, auxins and gibberellins have been shown to form complexes with lecithin (Wood et al . 1974; Wood and Paleg, 1974).

Gibberellins can also promote the secretion of alpha-amylase, beta 1-3 glucanase and protease as well as ions from aleurone layers (Chrispeels and Varner, 1967; Jacobsen and Varner, 1967) which is believed to operate via membrane bound vesicles, again suggesting an action of PGS on membrane function. It is well established that there is a relationship between stomatal aperture and ABA (Walton, 1980). ABA may have its effects on stomatal aperture by altering potassium fluxes across outer membrane. If PGS can penetrate the plasmalemma with relative facility then the presence of an outer membrane receptor would seem redundant.

Many developmental processes are accompanied or preceded by specific changes in enzyme activity. In some instances, this represents the synthesis de novo of those enzymes (Filner and Varner, 1967 ; Lewis and Varner, 1970; Grierson et al . 1985). For this reason, it is believed that the mode of action of PGS may involve the control of the production of mRNA and the de novo synthesis of enzymes.

1.3.2 Effects on gene expression

There are a number of reports on the effects of PGS on transcription and translation (For reviews see Jacobsen, 1977; Jacobsen and Higgins, 1978). Every PGS can, apparently, under the appropriate circumstances regulate the synthesis of RNA and protein. Several approaches have been taken to study this regulation. Originally PGS action was evaluated by measuring the level of total

RNA. The studies were based largely on the use of isolated nuclei (Cherry, 1976), isolated chromatin (O'Brien et al . 1968) or labelling and fractionation of total nucleic acids on columns of methylated albumin-kieselguhr (Key and Ingle, 1968). In this respect, auxins (Chen et al . 1975), gibberellins (Johri and Varner, 1968) and cytokinins (reviewed by Kende, 1971) have been shown to increase the rate of transcription whereas ABA (Pearson and Wareing, 1969) caused a reduction in transcription rate.

Effects on the activity of RNA polymerase in vitro have also been detected with every GS tested (Johri and Varner, 1968; O'Brien et al . 1968; Abeles, 1973; Kende, 1971; Walton, 1980). In addition, the effects of PGS on the activity of a number of enzymes which are synthesised de novo are well documented (Yomo and Varner, 1971; Abeles, 1973; Ihle and Dure, 1970; Kende, 1971; Grierson and Tucker, 1983).

The ultimate manifestation of expression of genetic information for a protein-coding gene is the appearance of the active protein. Many controls may act in concert to affect the ultimate synthesis of a protein. The regulation of gene expression by PGS may thus occur at any of several levels. In general, control of gene expression is possible at the level of transcription, post-transcription, translation and post-translation. The demonstration of the appearance of an enzyme does

not necessarily reflect an alteration in synthesis but may result from changes in the rate of turnover (Trewavas, 1976). Several methods have been used to distinguish between the levels of control of gene expression for a particular enzyme. The most widely used method has been the use of inhibitors of RNA synthesis (such as actinomycin D) and protein synthesis (such as cycloheximide). This method, however, has been criticised, since many inhibitors may have unwanted side effects (Trewavas, 1982).

Inhibitors can only give general information about the level of control of gene expression. This is particularly true of 'negative' results obtained with them. High concentrations of actinomycin D did not prevent the increase in activity of isocitrate lyase and malate synthase in germinating water melon cotyledons, whereas cycloheximide did prevent the increase (Hock and Beevers, 1966). In the absence of information on the possible side effects of inhibitors on this system, this result merely suggests but does not prove that the increases in enzymatic activity in isocitrate lyase and malate synthase are not dependent on transcription and that they are dependent on translation. Thus in the absence of additional data, dependence on protein synthesis cannot be interpreted in terms of de novo synthesis of enzyme protein. By contrast, the reverse situation was true for the increase in activity of ribonuclease activity in pea, which was not prevented

by cycloheximide even at highly toxic concentrations (Bryant, 1976). This change therefore does not seem to depend on protein synthesis and it is probably regulated post-translationally. This may also be the case for beta-1,4 glucan synthase, whose increase in activity was not prevented by actinomycin D or cycloheximide (Ray, 1973).

The availability of methods for assaying individual mRNA species has allowed a closer examination of PGS action at the molecular level. Thus, by using cell-free translation systems it has been possible to show direct effects of GS on particular mRNA species. Gibberellins have been shown to enhance the level of translatable alpha-amylase mRNA in aleurone layers (Higgins et al . 1976; Mozer, 1980) and to increase the amount of total polysomal RNA in castor bean seeds (Martin and Northcote, 1983). Likewise, auxins increase the level of total translatable mRNA in soybean hypocotyls (Zurfluh and Guilfoyle, 1982) and in pea stem (Theologis and Ray, 1982). ABA on the other hand has been shown to induce some mRNA species and to inhibit others in aleurone layers (Mozer, 1980) and to modulate other mRNA species in wheat (Williamson et al . 1985) and in rape embryos (Crouch et al . 1983). Cytokinins can induce the appearance of ribulose biphosphate carboxylase mRNA in pumpkin cotyledons (Parthier et al . 1982) and modulate some mRNA species in tobacco cell suspensions (Seyer and Lescure, 1984).

From this brief discussion, it is clear that information on the level of control of gene expression is not easily obtained. The more recent recombinant DNA methods do permit less ambiguous answers to this problems by enabling measurements of mRNA levels directly. Thanks to this methodology, it has been possible to study the activity of individual genes under different experimental conditions, that is, in the presence or absence of the inducing agent.

The cloning of DNA complementary to mRNA sequences (cDNA) generates specific probes which can be used to provide useful information about mRNA sequences and the genes encoding them. Complementary DNA clones have thus been obtained from several auxin regulated enzymes in pea (Theologides et al . 1985) and soybean (Kroner and Key, 1985). Clones for beta-1,3 glucanase, whose synthesis seems to be regulated by auxins and cytokinins (Mohnen et al . 1985) and alpha-amylase, regulated by gibberellins and ABA (Baulcombe and Buffard, 1983; Chandler et al . 1984), have also been obtained. Likewise, clones for some light regulated genes (Bedbrook et al . 1980; Dunsmuir et al . 1983), nitrate induced genes (Crawford and Davis, 1985) or storage proteins (Forde et al . 1983) have been obtained. This has permitted the study in more detail of the regulation of these genes.

Most of these studies involve the use of hybridization methods, using a radioactive probe with a complex

population of mRNA species. It should be mentioned, however, that measurements of this kind only assay the steady state level of transcripts complementary to the probe. They do not provide information on the rate of synthesis, turnover or cellular location of any of the mRNA's. Thus, although it seems most plausible that large increases in steady state levels are mediated mainly by an increase in the rate of transcription, it is possible that a decreased rate of turnover might account for some or all of the effect in any given case. On the other hand, these changes may be due to an increase in RNA stabilization. There is evidence that PGS can cause stabilization of previously formed mRNA (Gayler and Glasziou, 1969). RNA stabilization could also be associated with the suppression of ribonuclease activity by different PGS (Truelsen, 1967; Pilet, 1970; Srivastava and Ware, 1965).

The construction and introduction of chimaeric genes into the genomes of higher plants (Herrera-Estrella et al . 1984) may provide a useful tool with which to examine the expression of particular genes and the action of PGS and other stimuli at the molecular level.

It has been proposed (Darnell, 1982) that the regulation of gene expression occurs predominantly at the level of transcription. However several criticisms have been made against this idea as control of gene expression may occur at other levels (Trewavas, 1983). It is not yet clear whether controls other than transcriptional are

more important than transcriptional controls as has been suggested by Harris (1974). Control at levels other than transcriptional has been demonstrated in several instances (Ray, 1973; Barker *et al.*, 1974; Bryant, 1976). In this respect, Kroner and Key (1985) have suggested that auxin regulates the appearance of several mRNA species at the transcriptional as well as the post-transcriptional level.

1.4 ETHYLENE

Research on ethylene started last century with the work of Girardin on the effects of illuminating gas on plants, effects such as leaf and flower abscission and fruit ripening (for reviews on ethylene and its physiological effects see Ables, 1973; 1985). Since then and for many years the investigations on ethylene physiology were concerned primarily with the enhancement of fruit ripening by ethylene. The advent of gas chromatography made possible to measure this gas at physiologically significant levels (Burg and Stolwijk, 1959).

The biosynthetic pathway of ethylene has been elucidated (Adams and Yang, 1979) and methionine has been shown to be the precursor of ethylene (Lieberman and Mapson, 1964) and ACC an intermediate (Adams and Yang, 1979). Ethylene has been implicated in a number of developmental processes in plants (see Ables, 1973; 1985, for a review⁵). However, the exact way that ethylene regulates plant metabolism is unknown. It is

possible that ethylene plays a role as second messenger in plant development as there are several factors known to induce ethylene synthesis (Yang, 1980). Auxins, cytokinins, calcium, wounding and anaerobiosis can induce ethylene synthesis probably by regulating the activity of ACC synthase which is thought to be the rate controlling enzyme in ethylene biosynthesis (Yang, 1980).

1.4.1 Effects on Membranes

Since ethylene is more soluble in organic solvents than in water and since membranes contain large quantities of lipids it has been suggested that ethylene affects membrane permeability (Sacher, 1962). However, the evidence in this respect is not conclusive. Carbon monoxide, an ethylene analog does not share the solubility characteristics of ethylene but it has a rather similar physiological activity (Abeles, 1973). Fruit ripening, a developmental sequence controlled by ethylene, has long been associated with changes in membrane permeability (Sacher, 1962). However, these changes may be the result of ripening rather than a cause (Burg et al . 1964; Sacher and Salminen, 1969). Ethylene has also been found to have some effect on membrane permeability in avocado, banana and bean (Sacher and Salminen, 1969; Abeles, 1973). In addition, several investigators have found that ethylene increased or regulated the rate of enzyme secretion (a membrane-controlled process) from plant cells. This was

found to be the case for peroxidase (Ridge and Osborne, 1970), alpha-amylase (Eastwell and Spencer, 1982) and cellulase (Abeles and Leather, 1971). The data available do not support the idea of ethylene having a disruptive effect on membranes, but they do show that ethylene is implicated in secretory phenomena and the transport of materials through membranes.

1.4.2 Mode of action at molecular level

It is generally believed that ethylene acts primarily through a cellular receptor. This was first suggested by Burg and Burg (1965) who proposed that ethylene action involved the binding of ethylene and oxygen to the metal of a metalloenzyme and Beyer (1975) has suggested that ethylene metabolism to ethylene oxide is essential for ethylene action. Some binding of ethylene to plant tissues has been demonstrated in two instances (Sisler, 1979; Bengochea et al . 198¹~~0~~). The significance of this is still uncertain.

Valuable tools for studying ethylene action have been the observations that carbon dioxide (Burg and Burg, 1967), silver ions (Beyer, 1976) and hypobaric atmospheres (Burg and Burg, 1965) can all block ethylene effects.

Ethylene seems to be synthesized by all plant cells all the time, although the production rates may vary and are normally low (Abeles, 1973; 1985). For this reason, its ability to act as a regulator might be dependent on factors such as changes in the sensitivity of the tissue

(Trewavas, 1982; Abeles, 1985). Two possible examples are the processes of abscission (Abeles, 1967) and fruit ripening (Lyons and Pratt, 1964; Grierson et al . 1985) in which the response to ethylene seems to depend on the developmental stage of the tissue.

Direct and indirect approaches have been used in the study of ethylene action. The direct approach has been to examine the physical binding of ethylene to the plant (as mentioned before). The indirect approach has been to study ethylene mediated processes and, working backwards, try to learn more about the details of earlier events in the system. Since the observation that ethylene increased the activity of both invertase and protease in pineapples (Regeimbal and Harvey, 1927) there have been suggestions that the control of enzyme synthesis might play a role in ethylene action. Ethylene has been shown to regulate the activity of a number of enzymes associated with processes such as ripening, abscission, etc (Abeles, 1973; 1985; Lieberman, 1979). As RNA and protein synthesis inhibitors prevent the appearance of many of these enzymes, it has been suggested that the regulation involves synthesis de novo . This has been demonstrated to be the case with polygalacturonase in ripening tomato (Grierson et al . 1985) and cellulase in bean abscission zones (Lewis and Varner, 1970) and in ripening avocado (Christoffersen et al . 1984). In addition, ethylene modulates changes in the mRNA population in avocado

(Christoffersen et al . 1982), soybean (Zurfluh and Guilfoyle, 1982) and carrot (Nichols and Laties, 1984). In all these cases, transcriptional regulation by ethylene has been proposed, although ethylene may also have post-transcriptional and translational effects (Jacobsen and Higgins, 1978).

1.5 THE PROCESS OF ABSCISSION

1.5.1 General features

The term abscission is used to describe the process whereby various structures are shed from the parent plant (for reviews see Addicott, 1982; Sexton and Roberts, 1982). The process is characterized by cell wall breakdown and involves the active participation of the cells on either side of the fracture line (Sexton, 1976; 1979). Although virtually any aerial portion of a plant can be abscised, the process is limited in the majority of the cases to the shedding of leaves, bud scales, immature flowers, fruits and petals (Sexton et al . 1985). The point at which fracture will occur is probably genetically determined and takes place at predictable positions known as abscission zones which are found at the base of the organ to be shed (Addicott, 1982). Cell separation does not occur throughout the entire abscission zone but is limited to a discrete 1-3 cell wide "separation layer" at the distal end of the abscission zone (Gawadi and Avery, 1950; Halliday and Wangermann, 1972).

There is some evidence that cells in the separation

layer are cryptically differentiated (Osborne, 1982; Osborne et al . 1985) as they cannot usually be distinguished before abscission occurs. The time taken to complete the process is variable. In leaves 10-48 h are required (De la Fuente and Leopold, 1969; Sexton, 1979) whereas in flowers 1-4 h may be sufficient (Henry et al . 1974).

1.5.2 Methods for studying abscission

Although abscission has been studied in a number of plants, the system that has received most attention is the abscission of leaves in bean (Phaseolus vulgaris). As this is the experimental system concerned in this study, the discussion will be mainly confined to this system. It is known that plant tissues synthesise ethylene (Abeles, 1973; 1985) and that prior to natural shedding, rates of ethylene production in vivo are sufficient to accelerate abscission in many systems (Jackson and Osborne, 1970; Lipe and Morgan, 1973). As a result many workers have felt justified in adding 1-50 ppm of ethylene to synchronize and accelerate experimentally-induced abscission.

A useful experimental material to study leaf abscission has been an "explant" of the petiole region containing the abscission zone itself (Addicott et al . 1949)(Figure 1). The explants can be enclosed in a humid atmosphere and they will abscise in reasonable synchrony on ethylene treatment (Addicott et al . 1949). The progress of abscission is usually measured by the

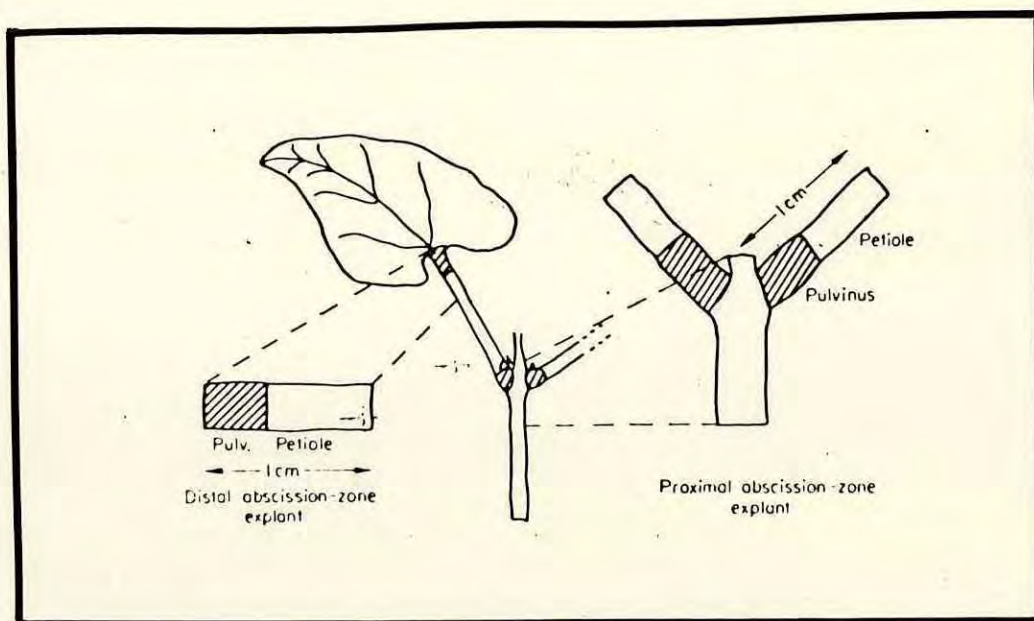


Figure 1 Diagram of a bean explant

Bean seedlings are grown for about two weeks and then the primary leaves and the stem are removed. The resultant material is called explant. There are two abscission zones in the bean seedling. The proximal abscission zone was employed in this study in to order to avoid contamination by leaf tissue which can result from using the distal abscission zone as experimental material. After the different incubations in air or ethylene, the abscission zones were excised (shaded area) and immediately frozen in liquid nitrogen and processed or stored at -70°C . The zones could be kept for several weeks in this condition.

"breakstrength" of the zone, which is the force required to cause a break in the abscission zone (Addicott et al . 1949; Durbin et al . 1981). With this technique, it has been relatively straightforward to determine the progress and the kinetics of the process. During abscission there is an increasing weakening of the zone, that is, a decline in breakstrength (Addicott et al . 1949; Craker and Abeles, 1969; Durbin et al . 1981). Evidence, primarily structural, has shown that cell separation and cell wall dissolution take place during abscission (Scott et al . 1967; Webster, 1973; Sexton, 1976). In addition, there are signs of increased metabolic activity in the zone (Addicott, 1970). There is an increase in respiration rate similar to the climateric of ripening fruits (Abeles and Gahagan, 1968; Marynick and Addicott, 1976) and an increase in the synthesis of RNA and proteins (Holm and Abeles, 1967; Abeles and Holm, 1967). The process seems to be sensitive to the supply of oxygen since concentrations below 20% retard abscission (Marynick and Addicott, 1976). All these changes precede and accompany the increased activity of the numerous enzymes that become conspicuous during abscission (see below).

1.5.3 Molecular basis of the abscission process

Changes in the activity of enzymes such as succinic dehydrogenase, malic dehydrogenase, acid phosphatase and peroxidase have been detected during abscission and they have been associated with the increased respiratory

activity of the tissue (Addicott, 1970). The observation that cell wall breakdown occurred during abscission was an indication that abscission was probably the result of increased enzymatic activity. For this reason, the greatest attention has, by far, been given to enzymes that might possibly be involved in the degradation of cell wall particularly cellulases and pectinases. In this respect Horton and Osborne (1967) found increased cellulase activity to be correlated with abscission in bean. Similar correlations were observed in cotton and Coleus (Abeles, 1969) and Citrus (Ratner et al. 1969).

Lewis and Varner (1970) established that the increase in cellulase activity in bean explants was the result of synthesis de novo. Later investigations disclosed the existence of several cellulase isoenzymes and showed that only one appeared closely correlated with abscission (Sexton et al. 1980). The substrate for assaying this enzyme in vitro is carboxymethyl cellulose and the trivial name cellulase has been adopted, though it is more properly described as a beta-1;4 glucan hydrolase. It is not known what the natural substrate for this enzyme is although cellulose and xyloglucans have been suggested (Sexton and Roberts, 1982).

Because cellulose is recognized as the major constituent of the fibrils of the primary and secondary cell wall (McNeil et al. 1984), it was assumed that cellulase

was required for cell separation (Abeles, 1969) and that increased cellulase activity promoted the cell disintegration that sometimes accompanies abscission (Horton and Osborne, 1967). There was also a close correlation between the increase in cellulase activity and the decline in breakstrength (Sexton et al . 1980). RNA and protein synthesis inhibitors which prevented abscission also inhibited the appearance of the enzyme (Abeles, 1969; Ratner et al . 1969). Abscission accelerators also increased both cellulase activity and the rate of breakstrength decline (Abeles, 1969; Cracker and Abeles, 1969; Ratner et al . 1969). Cellulase, thus, appeared to conform all the criteria for an abscission-controlling enzyme. However, these assumptions were questioned by the observations that during abscission there is little indication of cellulose breakdown in the primary wall (Sexton, 1976; 1979). The degradation seems to occur mainly in the pectic substances of the middle lamellae (Sexton, 1976; 1979). In addition, cellulase in Begonia only accumulated after the separation was essentially complete (Hanisch ten Cate et al . 1975). All these observations have to be reconciled with the finding that injection of cellulase antibodies into bean abscission zones stops cellulase accumulation and retards abscission whereas injection of pre-immune serum does not have any of this effects (Sexton et al . 1980). The involvement of cellulase in abscission,

therefore, remains in question.

Pectin methylesterase (Osborne, 1968) and polygalacturonase (Morre, 1968) have been shown to increase their activity during abscission. Their precise role in the process is still to be determined. In addition other hydrolases such as nucleases, proteases and phosphatases all increase during abscission (Cracker and Abeles, 1969). The significance of these changes is not really understood, but they have been interpreted as general symptoms of senescence (Cracker and Abeles, 1969).

1.5.4 Regulation of enzyme synthesis during abscission

It has been demonstrated that ethylene can accelerate abscission in vitro and in vivo since the levels of ethylene in abscission zones often reach the threshold necessary to accelerate abscission (Abeles, 1967; Addicott, 1970; Jackson and Osborne, 1970; Lipe and Morgan, 1973; Sexton et al . 1985). This has led to the hypothesis that ethylene is the natural regulator of abscission (Jackson and Osborne, 1970). This hypothesis has been questioned by Beyer (1975) who showed that ethylene can only induce abscission in vivo in certain conditions. He observed that the initial effect of ethylene in vivo was in the leaf blade. This caused the leaf to senesce. Senescence has been proposed to be a preparative step for abscission, making the cells in the abscission zone sensitive to ethylene (Burg, 1968;

De la Fuente and Leopold, 1968). In conclusion, the available evidence is only sufficient to indicate an intimate involvement of ethylene and abscission (Sexton et al . 1985). The response to ethylene is dependent on the sensitivity of the tissue (Jackson et al . 1973; Trewavas, 1976).

Auxins on the other hand, normally retard abscission (Addicott, 1970; Sexton and Roberts, 1982) but will accelerate it in certain conditions for instance if auxins are applied proximal to the abscission zone (Addicott and Lynch, 1951; Addicott, 1970). Many believe the abscission accelerating effects of auxins to be due to auxin induced ethylene formation (Osborne and Sargent, 1976). There seems to be an antagonistic action between auxins and ethylene during abscission. It has been suggested that the auxin status of the abscission zone is a major factor regulating its sensitivity to ethylene (Addicott and Lynch, 1951, Louie and Addicott, 1970). There is a clear correlation between the effects of ethylene, auxins and cellulase accumulation. Cellulase activity is increased by ethylene (Abeles, 1969; Lewis and Varner, 1970) and decreased by auxins (Horton and Osborne, 1967; Sexton et al . 1980). These observations are in complete agreement with the effects of ethylene and auxins on abscission. Thus, it seemed likely that ethylene and auxins regulated abscission by directly modifying the activity of enzymes in the abscission zones. Work with RNA and protein

synthesis inhibitors suggested that de novo synthesis of enzymes was a necessary requirement for abscission (Abeles and Holm, 1967; Holm and Abeles, 1967). Holm and Abeles (1967) also suggested that it was the ethylene-induced synthesis of mRNA that was essential.

However, ethylene can also stimulate abscission in other systems that are insensitive to transcriptional inhibitors (Henry et al . 1974; Hanisch ten Cate et al . 1975). Thus, ethylene may regulate abscission at different levels.

Cellulase is not the only enzyme in the abscission zone that responds to ethylene. Polygalacturonase (Riov, 1974) and uronic acid oxidase (Huberman and Goren, 1982) are also induced by ethylene but there is no evidence that they are directly related to abscission. However, in this respect, Lewis et al . (1974) were able to detect cell separation and protoplast formation in tobacco cell culture with a combination of cellulase and polygalacturonase

Although ethylene and auxin seem to have the most crucial effects on abscission, there are a number of biological and physical factors (Addicott, 1982) that can affect abscission. However it is not known whether they have a direct effect on enzyme activity.

1.6 MECHANISMS OF DEFENSE IN PLANTS

1.6.1 General remarks

One of the consequences of leaf abscission is the production of a scar in the stem which may be

susceptible to infection by pathogens. Clearly, there should be mechanisms by which plants stop or prevent the spreading of disease in this and other instances. Higher plants have evolved a large variety of mechanisms of resistance against infection by pathogens. In a broad sense, the mechanisms are located on plant cell surfaces and/or the cytoplasm. By these mechanisms, plants are able to prevent, restrict or retard the development of disease. Defense mechanisms in plant have been reviewed by Bell (1981) and Sequeira (1983).

1.6.2 Mechanisms of defense at the cell wall level

Plant cell walls were originally considered as efficient, yet passive barriers against pathogens. The discovery that changes in cell wall composition, lignins, proteins or glycoproteins do occur upon infection gave another view of the role of cell surface in pathogenesis. Lignification, accumulation of hydroxyproline rich glycoproteins and inactivation of pathogen degrading enzymes will be considered in turn.

1.6.2.1 Lignification

Formation of lignin may be an important factor in preventing the development of pathogens (Friend et al . 1973; Ride, 1975). Vance and Sherwood (1976) have shown a localized lignin formation in the middle lamellae of the parenchyma cell walls and around the penetrating fungus. They also showed that cycloheximide inhibited this increased lignin deposition, which suggested that enzyme formation was involved. This

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result was confirmed by Hahlbrock and co-workers (Lawton et al . 1983; Ryder et al . 1984), who showed an increase in the rate of synthesis of up to four enzymes of phenyl propanoid biosynthesis on challenge by fungal elicitors (see below).

As lignin acts as a hydrophobic filler in the cell wall displacing water (Northcote, 1972), it might be that fungal progress is stopped by the exclusion from the cell wall of water, which would then prevent the spread of the infection. Along with lignin, plant cell walls contain phenolic compounds (McNeil et al . 1984) which could contribute to the defense of plants through their linkage with oligosaccharides, thus protecting these carbohydrate polymers from degradation by pathogen hydrolases.

1.6.2.2 Accumulation of hydroxyproline-rich glycoproteins (HRGP)

At least three classes of HRGP exist in plants (see McNeil et al . 1984, for a review). These are (1) the extensins, (2) the arabinogalactan-proteins and (3) the Solanaceae lectins. Extensins are found most abundantly in the cell walls of dicotyledonous plants and are assumed to play a role in the structure of plant cell walls (Cassab et al . 1985). They may, therefore, be important in controlling growth. Extensins also accumulate in plant cell walls on wounding (Chrispeels et al . 1974) and pathogen attack (Esquerre-Tugaye and Lamport, 1979), therefore, they may be involved in

defense. Recently Varner and co-workers have shown that in bean and carrot the level of mRNA's coding for HRGP changes in response to biological stress (Showalter et al . 1985; Chen and Varner, 1985).

1.6.2.3 Inactivation of pectolytic enzymes of pathogens

These enzymes, believed to be of key importance in numerous infection processes, may be inactivated by inhibitors. Plants have within their walls proteins that can inhibit specifically the wall-degrading enzymes secreted by the pathogen (Albersheim and Anderson, 1971; Fisher et al . 1973). Protein inhibitors probably represent a general rather than a specific mechanism for resistance. The inhibitors may be synthesised in response to pathogen attack (Albersheim and Anderson, 1971).

1.6.2.4 Production of cell wall fragments

Short oligosaccharides have been shown to induce plant tissues to synthesise phytoalexins (Darvill and Albersheim, 1983). These molecules have been called elicitors and can be of plant origin (Nothnagel et al . 1983) or fungal origin (Ayers et al . 1976). Elicitors can change the metabolism of receptive plant cells so that the enzymes responsible for phytoalexin synthesis are synthesised de novo (Hahlbrock et al . 1981; Lawton et al . 1983; Ryder et al . 1984). The means by which elicitors activate the expression of specific genes is not understood. Elicitors can also

activate the synthesis of some hydrolytic enzymes involved in defense (see below). Some glycoproteins (De Wit et al . 1980) and chitosan (Walker-Simmons et al . 1983) have also been shown to have some elicitor activity. The physiological significance of this is unclear.

1.6.3 Mechanisms of defense at the cytoplasm level

1.6.3.1 Production of proteinase inhibitors

Proteinase inhibitors are found in many seeds or other storage organs, sometimes in high concentration and also in aerial tissues of plants (Ryan, 1973). They are usually proteins having a mw under 50 kD and more commonly under 20 kD. Interactions between proteases and plant proteolytic inhibitors led Ryan (1978) to hypothesize that the inhibitors could be a mechanism of disease resistance. This hypothesis is supported by the fact that proteolytic inhibitors accumulate in potato and tomato plants on damage by beetles (Green and Ryan, 1972) or fungi (Peng and Black, 1976). This accumulation also occurs after wounding. In all reported cases, infection or wounding of a single bottom leaf induces proteinase inhibitor activity in undamaged upper leaves, suggesting the presence of a "signal" which has been called proteinase inhibitor inducing factor. It has been suggested that this factor may be an oligosaccharide of plant origin, related to the cell wall (Ryan, 1978).

1.6.3.2 Synthesis of phytoalexins

These are compounds with antibiotic properties of low mw

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that are both synthesised by and accumulated in plants after exposure to different stresses (Darvill and Albersheim, 1984). Evidence has been provided that phytoalexins not only accumulate at the site of infection but that they do so, following penetration by the pathogen, quickly enough and in sufficiently high concentration to inhibit the growth of fungi (Bailey, 1974) and bacteria (Lyon and Wood, 1975). Accumulation of phytoalexins seems to result from de novo synthesis of enzymes of the phytoalexin biosynthetic pathway (Hahlbrock et al . 1981; Lawton et al . 1983; Ryder et al . 1984). Fungal or plant elicitors can induce the synthesis of phytoalexins (Darvill and Albersheim, 1983). It is believed that phytoalexins have a static rather than a toxic effect by altering the structural integrity of cell membranes. Their action is not discriminatory and thus would explain the toxic effect that phytoalexins have been observed to have sometimes on higher plants (Darvill and Albersheim, 1984).

1.6.3.3 Hypersensitivity This mechanism of defense is characterized by disorganization, browning and death of cells at the infection sites (Varns et al . 1971). It has been suggested that the growth of pathogens is restricted in cells which react in a hypersensitive manner and the infective agents are damaged or even killed in necrotic tissues by the substances liberated by the plant (Kiraly et al . 1972). The production of

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phytoalexins by infected plants has been associated with the hypersensitivity reaction (Kiraly et al . 1972).

1.6.3.4 Synthesis of lytic enzymes as possible defense mechanisms against fungi

Two glucan hydrolases, chitinase and beta-1,3 glucanase have received special attention as chitin and beta-1,3 glucans are components of many fungal cell walls (Bartinicki-Garcia, 1968) In vivo lysis of fungal hyphae caused by chitinase and beta-1,3 glucanase in tomato have been reported by Pegg and Vessey (1973) and Pegg (1976). Wargo (1975) extracted beta-1,3 glucanase and chitinase from the phloem of stems and roots of different perennial woody plants. The enzymes hydrolyzed the hyphal wall of pathogens attacking forest trees. The presence of these enzymes in healthy trees suggests a protective mechanism against fungal pathogens. Oligosaccharides of beta-1,3 glucan and chitin are active elicitors. More recently Netzer and Kritzman (1979) showed that in muskmelon the increase in beta-1,3 glucanase activity provided a potential defense mechanism against fungi. Inoculation of fungi or inoculation of laminarin, a beta-1,3 glucan, induced an increase in activity of the enzyme. A correlation was found between the limited spread of the pathogen and the induction of the enzyme after pretreatment with laminarin. In contrast, Rabenantoandro et al (1976) found that in melon the increased beta-1,3 glucanase activity upon infection was involved in the infective

mechanism of the pathogen whereas chitinase activity was implicated in defense.

Chitinase and beta-1,3 glucanase have also been shown to be induced by fungal elicitors (Mauch et al . 1984; Metraux and Boller, 1985). In the case of chitinase there was a 600-fold increase in activity after induction in cucumber leaves. Mauch et al (1984) also showed that the induction of chitinase by elicitors was systemic, although the increase in activity in uninfected areas of the plant was lower than that of infected areas.

Exogenous ethylene has been shown to induce the synthesis of these hydrolases (Abeles et al . 1970; Rabenantoandro et al . 1976; Boller et al . 1983). As stress ethylene is formed as a result of a pathogen attack, wounding or other stresses (Yang, 1980; Konze and Kwiatowski, 1981), it was suggested that chitinase and beta-1,3 glucanase were induced by this stress ethylene. However, several studies seem to indicate that stress ethylene has little importance in the induction of biochemical defenses against pathogens (Paradies et al . 1980; Boller et al . 1983; Mauch et al . 1984). Suppression of ethylene synthesis by inhibitors did not prevent the induction of the two enzymes by fungal elicitors. Mauch et al (1984) have concluded that ethylene and elicitors are separate and independent stimuli for the induction of the two hydrolases and that stress ethylene production is only a symptom but not a

necessary signal for the induction. Stress ethylene synthesis is stimulated for a short period only (Yang, 1980; Konze and Kwiatowski, 1981). Thus ethylene may not be present long enough for induction which in the case of chitinase requires a continuous presence of ethylene (Böller et al . 1983). Chitinase has also been shown to have lysozyme activity (Boller et al . 1983). A striking result of these studies is that chitinase seems to be induced more strongly, about 60-fold, by fungal elicitors than by ethylene (Metraux and Boller, 1985). Chitinase has been detected in the leaves of bean, tomato, pea, cucumber, cotton and (Abeles et al . 1970; Boller et al . 1983; Metraux and Boller, 1985), in several seeds (Powning and Irzykiewicz, 1965; Wadsworth and Zikakis, 1984) and wheat germ (Molano et al . 1979). Ethylene has only been shown to induce the synthesis of chitinase in plant leaves. The evidence indicates an intimate involvement of chitinase as an inducible enzyme against fungal and, perhaps, bacterial pathogens. The observation that chitinase can be induced by ethylene in bean abscission zones makes this system a useful model for studying the action of ethylene.

1.7 CONCLUSIONS

The preceding sections have dealt with plant development, the central role that PGS have in it and some mechanisms of defense that plants have against pathogens . PGS can modulate gene expression in plants and therefore the study of their action is fundamental

to our understanding of the way plant development and morphogenesis take place.

The process of abscission is an excellent model system in which to study the control that PGS may exert upon gene expression. The use of explants as experimental material was a significant advance in methodology as the explants can be treated with ethylene, an accelerator of abscission, and made to abscise in reasonable synchrony thus providing reproducible material. New species of mRNA are synthesized in response to ethylene which makes this process an interesting one to study in more detail.

Plants show an innumerable array of mechanisms of defense against pathogens. These mechanisms may act individually or collectively so that the final result is an effective protection against invasion. Many of these responses of plants to the demands of the environment are regulated at the gene level, with new proteins being synthesised in response to stress. The mechanism of defense in plants need not necessarily be similar to those of animals as the demands that the environment poses upon them may be different.

1.8 AIMS AND APPROACH OF THE PROJECT

The general aim of this study was to contribute to a better understanding of the way PGS regulate gene expression in plant development. The induction of chitinase during the process of ethylene-accelerated abscission has been used as a model system.

When this project commenced current work in this

laboratory was concerned with the process of understanding the molecular basis of abscission in Phaseolus vulgaris leaf explants. Using in vitro translation, it had been shown that several species of mRNA giving translation products of 42 kD, 32 kD and 17 kD accumulated greatly after ethylene treatment. The 42 kD product had been tentatively identified as cellulase using antibodies.

During the course of this work, Boller et al (1983) published a paper on the induction of chitinase by ethylene in leaves of Phaseolus vulgaris. This enzyme was a prominent species making up to 4% of the leaf protein and had a mw of 30 kD on SDS-PAGE. It, thus, seemed probable that the 32 kD translation product in the abscission zone was an unprocessed chitinase precursor. The following facts supported this conclusion:

- 1) The 2 kD discrepancy in mw could be due to the presence of a signal peptide in the in vitro translation product. This size for a signal peptide is well within the range reported in the literature (Wickner, 1980; Kriel, 1981).
- 2) The 32 kD translation product appeared at 6 h of ethylene treatment and so did chitinase activity in leaves.
- 3) The 32 kD was a major species amongst the translation products (approximately 8% of the total) of abscission zones. Chitinase was also an abundant polypeptide in

leaves, making up about 4% of the total leaf protein (Boller et al . 1983).

The purpose of the work presented in this thesis was to attempt to establish if this 32 kD product was a chitinase precursor. A outline of the proposed programme involved is detailed below:

- 1) To establish by enzymatic assay that chitinase was present in abscission zones as well as in leaves and to follow its activity on induction of abscission by ethylene.
- 2) To purify chitinase and prepare monospecific antibodies against it.
- 3) To use the chitinase antibodies to immunoprecipitate the 32 kD translation product.
- 4) To use the antibodies in immunoblot experiments to try to correlate the increase in enzymatic activity with an increase in chitinase antigen.
- 5) To attempt to process the 32 kD product into the mw of chitinase (30 kD) using microsomal membranes.
- 6) To attempt to obtain a clone containing chitinase sequences and use it to quantify the levels of chitinase mRNA. This information would then be correlated with that obtained from points 1, 4 and from in vitro translation studies.

The experimental system used in this study involved the use of the proximal abscission zone from the primary leaves of bean (Phaseolus vulgaris). Small segments or explants containing the abscission zone itself were

obtained from 2-week old seedlings and made to abscise by treatment with ethylene. The explants would abscise slowly, if left, a process which is accelerated by inclusion of 50 ppm of ethylene. This acceleration causes that the explants abscise in reasonable synchrony providing a useful and reproducible experimental material. The result of the experiments using explants can be extrapolated to the in vivo situation, since in over 35 years of research using explants as experimental material no major differences in the biology of the two systems have emerged. It is claimed that the changes which bring about explant abscission are ~~xxxxx~~ similar to those that occur naturally (Sexton and Roberts, 1982). For instance, in those cases where natural abscission is preceded by senescence, the auxins flux through the abscission zone declines in concert with a rise in ethylene production. A similar situation may occur in the explants. Here auxin levels in the zone are believed to fall as a result of the removal of the subtending organ and ethylene levels are elevated exogenously.

A major part of this study was concerned with the analysis of proteins synthesised in vivo and in vitro. The method employed for this analysis was SDS-polyacrylamide gel electrophoresis. By this method, total cellular protein can be examined and the range, extent and nature of protein changes can potentially be indicated. Furthermore, information can be obtained on

the amount and rate of synthesis of proteins that occur even in low concentrations. In addition, it can provide a means to isolate proteins. Polyacrylamide gels were, therefore, the method of choice as an initial means of examining proteins.

Chitinase induction during abscission was examined by studying the increase in enzymatic activity, enzyme protein and mRNA. A correlation was then drawn between them. For evaluating the changes in variety and concentration of different mRNA species, the cell-free protein synthesising system of wheat germ was used. This is a very sensitive method for studying new species of mRNA being synthesised.

In order to have a preliminary indication as to the potential involvement of transcriptional control, attempts were made toward the cloning of chitinase. The availability of defined gene probes is an essential prerequisite for studying the regulation of gene expression. Reasons for investigation of particular aspects of the work are described at the beginning of the relevant chapter.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant Material

Beans (Phaseolus vulgaris cv. Red Kidney) were obtained from Roy Sexton (Stirling).

2.1.2 Chemicals

General chemicals (Analar grade or Sigma grade) were purchased from British Drug Houses (BDH) or Sigma Chemical Co.

Chemicals obtained from other sources were:

Bacto Agar, tryptone, yeast extract: Pifes Ltd.

Cesium Chloride: Fison Scientific Apparatus.

Coomassie Brilliant Blue R: Raymond A. Lamb Ltd.

2'deoxy-N-5'triphosphates: Boehringer Mannheim.

Tri-iso-propylnaphtalene sulfonic acid, sodium salt (TNS): Eastman Chemicals.

Triton X 100: Hopkin and Williams, Ltd.

2.1.3 Enzymes

Restriction Enzymes were from Bethesda Research

Laboratories, Boehringer Mannheim or P & S

Biochemicals Ltd.

Creatine Phosphokinase (CPK): Boehringer Mannheim.

T4 Polynucleotide Kinase: P & S Biochemicals Ltd.

DNA Polymerase I (E. coli); New England Biolabs.

All enzymes were stored at -20°C.

2.1.4 Radio isotopes

Purchased from Amersham International, Ltd.

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alpha-³²P-dCTP triethylammonium salt (in a stabilized aqueous solution) 400 Ci/mmol, 10 uCi/ul.

gamma-³²P-ATP triethylammonium salt (in a stabilized aqueous solution) 7000 Ci/mmol, 10 uCi/ul.

L-³⁵S-methionine (in aqueous 20 mM potassium acetate, 1 mM DTT solution) 1150 Ci/mmol, 5 uCi/ul.

Tritiated amino acid mixture (All in a stabilized solution containing 2% ethanol, at 1 uCi/ul).

L-³H-Leucine 120-190 Ci/mmol;

L-³H-Lysine 75-100 Ci/mmol;

L-³H-Phenylalanine 100-130 Ci/mmol;

L-³H-Proline 100-130 Ci/mmol;

L-³H-Tyrosine 70-100 Ci/mmol;

¹²⁵I-Protein A, 2 uCi/ul was a gift of Dr. D. Apps, Biochemistry Department, University of Edinburgh.

2.1.5 Film

X-Ray film: DuPont Cronex 4

Negative film: Kodak Technical Pan and Polaroid 667.

2.1.6 Supplies

Nitrocellulose: type BA 85, 0.22 or 0.45 um pore size: Schleicher and Schuell.

Nylon membranes: Hybond N, Amersham.

3 MM filter paper: Whatman.

Saran Wrap: Dow Chemical Co.

Oligo dT Cellulose: Pharmacia Biotechnology

2.1.7 Bacteria and Phage

The Phaseolus vulgaris genomic library was a gift from J.L. Slighton (Agrigenetics, Madison,

Wisconsin);

E. coli DH1 was from the same source.

E. coli LE 392 was a gift from D. Baulcombe
(PBI, Cambridge)

E. coli HB 101 was a gift from P. Isaac (Botany
Department, Edinburgh)

2.1.8 Buffers

Alkaline SDS : 0.2 N sodium hydroxide, 1% ^(w/v) SDS.

CT : 1 mM CDTA, 10 mM Tris base pH 8.

"Chloroform" : Solution of chloroform:isoamyl
alcohol (24:1, v/v).

DEF : 12% ^(w/v) Ficoll, 50 mM EDTA pH 8, 0.07% ^(w/v)
bromophenol blue, 0.07% ^(w/v) xylene cyanol.

100X Denhardt's : 2% ^(w/v) BSA, 2% ^(w/v) Ficoll type 400, 2% ^(w/v)
Polyvinyl pyrrolidone type 40 (Denhardt, 1966).

Hot SDS : 5% ^(w/v) SDS, 50% ^(v/v) glycerol, 0.1 M EDTA, 0.2% ^(w/v)
bromophenol blue.

IPDB : 50 mM Tris base, 190 mM sodium chloride,
1.25% ^(v/v) triton X-100, 6 mM EDTA pH 7.5.

IPWB : 50 mM Tris base, 150 mM sodium chloride,
0.1% ^(v/v) triton X-100, 0.2% ^(w/v) SDS, 5 mM EDTA pH 7.5.

2X Kinase : 0.1 M Tris base, 25 mM magnesium
chloride, 12 mM DTT, 10% ^(v/v) glycerol, 1 mM
spermidine.

10X MOPS : 0.2 M MOPS, 50 mM sodium acetate, 10 mM
EDTA, pH 7.

10X NT : 0.5 M Tris base, 0.1 M magnesium sulphate,
1 mM DTT, 0.5 mg/ml Bovine serum albumin (BSA)
pH 7.2.

PBS : 20 mM sodium phosphate buffer pH 7.2, 150 mM
sodium chloride 0.02 sodium azide.

'Phenol' : Phenol containing 10% (v/v) redistilled
m-cresol, 0.1% (w/v) 8-hydroxyquinoline and
saturated with 0.1 M Tris base pH 8.5.

'Phenol-Chloroform' : A 1:1 mixture of the two previous solutions.

Plasmids high salt buffer : 3 M potassium acetate, 1.8 M formic acid.

Plasmids lysis buffer : 50 mM glucose, 10 mM cyclohexane diaminetetraacetic acid (CDTA), 25 mM Tris base pH 8.

Prehybridization buffer : 6X SSC, 5X Denhardt's solution, 5% ^(w/v) SDS, 20 ug/ml denatured salmon sperm DNA. ^(w/v)

10X Restriction enzymes buffer : 0.1 M Tris base pH 7.5, 0.5 M sodium chloride, 0.1 M magnesium chloride.

RNA grinding buffer : 0.1 M Tris base pH 8.5, 6% ^(w/v) Para-amino salicylic acid (PAS) (w/v), 1% Tri-propylnaphthalenesulphonic acid (TNS) (w/v).

Southern denaturing solution : 0.8 M sodium chloride, 0.4 M sodium hydroxide.

Southern neutralising solution : 1.5 M sodium chloride, 0.5 M Tris base pH 7.5.

20X SSC : 3 M sodium chloride, 0.3 M sodium citrate, pH 7.

STE : 10 mM Tris base, 100 mM sodium chloride, 1 mM EDTA, pH 8.

10X TAE : 40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA, pH 8.

TPE : 0.8 M Tris base pH 8, 0.2 M sodium orthophosphate 0.02 M EDTA.

WG Buffer : 20 mM Tris base PH 7.6, 120 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT.

WTBB : 0.05% ^(v/v) Tween 20 and 0.02% ^(w/v) sodium azide in PBS.

2.2 METHODS

2.2.1 Plant Material

Seeds of P. vulgaris were grown in John Innes compost in a greenhouse at 26°C under natural light.

When the primary leaves were approaching full expansion (about two weeks old) plants were harvested. Stems were severed at ground level, washed in distilled water and cotyledons removed. The axes were placed in beakers with water in controlled atmosphere cabinets. The cabinets had a mixture of 50 ppm of ethylene in air flushing through at 15 l/min. Groups of plants were removed at different times and leaves and abscission zones sectioned and immediately frozen in liquid nitrogen. A similar series of air controls ^{was} ~~were~~ also taken. The frozen tissue was used immediately or stored at -80°C .

2.2.2 Chitinase extraction and purification

The method used was that of Boller et al. (1983). All steps were carried out at 4°C . Tissue was homogenized with a mortar and pestle or with a MSE "Atomix" homogenizer at full speed for 1-2 min in 2 volumes of 0.1M sodium citrate pH 5 containing 10 mM n-acetyl-L-cysteine. The slurry was filtered through two layers of cheese cloth and centrifuged ($15,000 \times g$, 15 min). The supernatant of this crude homogenate was saved and used as a source of enzyme. This supernatant was incubated for 20 min at 50°C . The precipitate was removed by centrifugation as before and ammonium sulphate added to the supernatant to 60% saturation. The mixture was stirred for 1 h at 4°C and the precipitate collected by centrifugation and redissolved in 10 mM sodium acetate buffer pH 5.5. Sodium bicarbonate was added to a final concentration of 20 mM and the pH

4

raised to 8.4 with 0.1M sodium hydroxide. Chitinase was purified by using a column of regenerated chitin (Molano et al . 1977).

The regenerated chitin was prepared by acetylation of chitosan with acetic anhydride. Chitosan (1g) was first dissolved in 20 ^{ml} ~~volumes~~ of 10% acetic acid 90 ^{ml} ~~volumes~~ of methanol were added and the solution filtered through glass wool. 1.5 mls of acetic anhydride were added to the filtrate and the solution left to solidify at room temperature for 15 min. Once solidified, the gel was covered in methanol and ground in the atomix for 5 min at full speed. The suspension was washed to neutrality with water in a sintered-glass funnel. The chitin was resuspended in 0.02% sodium azide to a concentration of 15 mg/ml (dry weight/vol).

The column (10 x 1 cm) was packed with the chitin, equilibrated with 100 mls of 20 mM sodium carbonate buffer pH 8.4 and washed with 100 mls of 20 mM sodium acetate buffer pH 5.5. The sample was loaded on to the column and fractions (3 mls) were assayed for chitinase activity, pooled, dialyzed overnight in three changes of 0.1 M sodium citrate buffer pH 5 and stored at 4°C. Chitinase was stable for up to four months at 4°C.

2.2.3 Estimation of protein concentration

This was carried out according to the method of Bradford (1976) as modified by Bearden (1978). This rapid and sensitive assay relies on the binding of protein to coomassie brilliant blue G-250 due to the interaction of

the dye with the amino groups of proteins (Fazekas de St. Gro~~th~~ et al . 1963). Binding causes conversion of the red form of the dye to the blue form with a corresponding shift in the absorption maxima of the dye from 465 to 595 nm.

2.2.3.1 Protein reagent Coomassie brilliant blue G-250 was dissolved in 85% (w/v) orthophosphoric acid at a concentration of 1 mg/ml. This was diluted fivefold with distilled water, filtered twice in Whatman No. 1 filter paper and stored at room temperature in a dark bottle. This solution was replaced every six weeks. The protein solution to be assayed was mixed with an equal volume of the protein reagent (1.5 mls final volume). The absorbance at 595 nm was measured after 5 min and before 1 h of the addition of the reagent. The amount of protein in the sample was determined by comparison with a calibrated curve prepared using BSA.

2.2.4 Chitinase assay

A radiometric assay was used (Molano et al . 1977). This assay relies on the liberation of diacetyl-chitobiose (soluble in water) by chitinase from radioactively labelled chitin (insoluble in water). The radioactive substrate was a kind gift of Tom Boller (Basel).

The reaction mixture in a microcentrifuge tube consisted of enzyme, 1.5 mg of tritiated chitin (0.16uCi/mg) and 20 mM sodium phosphate buffer pH 6.3 in a final volume of 0.25 ml. The reaction was stopped after 0.5 h with an

equal volume of 10% TCA. After centrifugation (12,000 x g, 10 min) the radioactivity of 0.25 ml of the supernatant was determined in 5 mls of 0.7% butyl-PBD in a 1:1 mixture of toluene and triton X-100 (v/v). A unit was defined as that amount of enzyme releasing 1 nmol of chitobiose per minute at 37°C. A standard curve relating amount of enzyme to product formation was employed for calculation of specific activities. As the reaction of chitinase with its substrate does not yield a straight line, a dilution series of the enzyme was prepared. The initial slope was used for calculation of enzyme units (see figure 18).

A viscometric assay was also developed (Otakara, 1961). Glycol chitin was prepared according to Araki e Ito (1975) using glycol chitosan as starting material. Glycol chitin in 50 mM sodium phosphate buffer (0.3% w/v) was incubated with an appropriate amount of enzyme at 30°C and the decrease in viscosity measured in an Ostwald viscometer. A unit was defined as that amount of enzyme causing a 50% decrease in viscosity in 1 min.

2.2.5 SDS-Polyacrylamide Gel Electrophoresis of proteins (PAGE)

PAGE was carried out by a modification of the method of Laemmli (1970). Slab gels (0.9 mm thick) were employed, using two glass plates, 23 x 18 x 0.3 cm and 21 x 18 x 0.3 cm separated by perspex spacers lightly coated with vacuum grease. The assembled glass plates were held in position by perspex clamps.

2.2.5.1 Stock buffers and solutions

Acrylamide solution : 30% (w/v) Acrylamide, 0.8% (w/v) bisacrylamide, stored in 20 mls bottles at -20°C.

4X Separating gel buffer : 1.5 M Tris, pH 8.8, 0.4% SDS stored at 4°C.

4X Stacking gel buffer : 0.5 M Tris pH 6.8, 0.4% SDS, stored at 4°C.

Electrodes buffer : 25 mM Tris Base, 192 mM glycine, 0.1% SDS, pH 8.2, freshly prepared.

2X sample buffer : 125 mM Tris, 21% Ficoll (type 400,000), 0.4 M DTT, 5% SDS, pH 6.8, stored at -20°C.

Bromophenol Blue : 0.2% in water. 0.012% final concentration per sample.

Staining solution : 0.2% Coomassie brilliant blue R 250 in 50% methanol, 7% (v/v) glacial acetic acid.

Destaining solution : 35% (v/v) ethanol, 8% (v/v) glacial acetic acid.

2.2.5.2 Discontinuous polyacrylamide gel composition

| | |
|-------------------------------------|-------------------------------------|
| 5% Stacking gel (2.5 cm long) | 12% Separating gel (16 cm long) |
| 5% (w/v) Acrylamide | 12% (w/v) Acrylamide |
| 0.13% (w/v) Bisacrylamide | 0.32% (w/v) Bisacrylamide |
| 0.125 M Tris base pH 6.8 | 0.375 M Tris base pH 8.8 |
| 0.1% SDS | 0.1% SDS |
| 0.1% (v/v) TEMED | 0.1% (v/v) TEMED |
| 0.05% (w/v) Ammonium persulphate | 0.05% (w/v) Ammonium persulphate |

Typically 12% SDS-gels were used to examine polypeptides. Gels were electrophoresed at a constant current of 20 mA (350 v maximum voltage) until the bromophenol blue reached the bottom of the gel. Gels

were fixed and stained for 0.5 h with shaking in three volumes of staining solution and destained in several changes of destaining solution. When required, gels were prepared for fluorography with En^3Hance (New England Nuclear). After destaining, gels were incubated for 1 h in four volumes of En^3Hance and for 0.5 h in distilled water with stirring. Gels were then dried on to 3 MM paper under vacuum at 70°C.

Dried gels were exposed to prefogged Cronex 4 X ray film (Bonner and Laskey, 1974). Prefogging was achieved by exposing the film, at a distance of about 20 cm, to a single 1/1000 second flash from a Sun Pak GT 32 flashgun covered with an Ilford S 902 filter and this in turn covered with Whatman No. 1 filter paper acting as a diffuser. The flashed side of the film was put in contact with the gel and exposed at -80°C for between one and two weeks.

The following proteins were used as molecular weight markers:

| | |
|---------------------------|--------|
| Bovine serum albumin | 68,000 |
| Catalase | 60,000 |
| Aldolase | 40,000 |
| Carbonic anhydrase | 29,000 |
| Soybean Trypsin inhibitor | 21,000 |
| Myoglobin | 17,200 |
| Lysozyme | 14,300 |

2.2.6 Protein transfer onto nitrocellulose filters

The method used was a modification of that of Towbin et al . (1979). All operations were carried out at room temperature. After electrophoresis, gels were equilibrated in transfer buffer (192 mM glycine, 25 mM

Tris base, pH 8.2, 20% methanol) for 1 h. Gels were placed on a presoaked sheet of nitrocellulose (0.22 μ m, pore size) or nylon membrane, cut to fit the gel. Methanol was excluded when transfer was performed on to nylon membranes. The assembly was sandwiched first between two sheets of 3 MM paper, then between two scouring pads and then between two plastic grids. Air bubbles were removed at all steps. The cassette was inserted into a tank filled with transfer buffer, the membrane facing the anode. Proteins were transferred using a Trans-blot cell (Bio-Rad Laboratories). Transfer took place overnight at 30 v, 0.1 A. After the transfer, additional binding sites on the membrane were blocked by incubation with WTBB at 42°C for 16-18 h.

Membranes were incubated with chitinase antiserum at a dilution of 1:500 in 0.05% Tween 20 in PBS, overnight. Chitinase was detected on the membrane by incubation with 125 I-Protein A at 200,000-500,000 cpm/ml of PBS for 1 h. Membranes were washed in 0.05% Tween 20 in PBS per 0.5 h (five times), dried and exposed to X-ray film at -80°C.

2.2.7 Preparation of antibodies to chitinase

Male New Zealand white rabbits (from Hacking and Churchill, Ltd) of 3 kg were injected subcutaneously behind the shoulder blade near the rear of the rib cage with 100 μ g of pure chitinase in 0.25 ml of PBS thoroughly mixed with an equal volume of Freund's complete adjuvant. A further subcutaneous injection was

made in a similar way after three and six weeks, except that incomplete adjuvant was used. The rabbits were bled 10 days later. The blood was left at room temperature for 90 min, the clot removed by centrifugation (6000 x g, 15 min) and the serum stored in small aliquots at 4°C. Sodium azide (0.02%) and trasylol (50 units/ml) were added to the serum.

2.2.8 Immunodiffusion and Immuno-electrophoresis

The homogeneity of the antigen and the specificity of the antiserum were tested by experiments of immunodiffusion in 1.2% agarose in 0.1M citric acid-phosphate buffer pH 7 (Clausen, 1971) and immuno-electrophoresis in 1% agarose in 0.05 M barbital buffer pH 8.2 at 4 V/cm (Clausen, 1971).

2.2.9 In vivo labelling of abscission zone chitinase

15 days old bean seedlings were used. Leaves and stems were removed, leaving an 'explant' containing the abscission zone and a section of the petioles and stem of about 1 cm each. The explants were cut transversally, soaked in 0.5% sodium hypochlorite for 10 min, rinsed with distilled water and placed, cut surface up on 2% agar in sandwich boxes covered with wet muslin. The explants were then incubated in 50 ppm ethylene for 16 h. 0.5 uCi of ^{35}S -methionine in 10 ul of water was applied to the cut surface of each abscission zone and the ethylene treatment continued for 12 h. A further 0.5 uCi was applied to each zone as before and a final ethylene treatment for 12 h was given.

The zones were isolated and frozen in liquid nitrogen. A crude homogenate was prepared by grinding the tissue in two volumes of 0.1 M sodium acetate buffer pH 5 and centrifuged at 15,000 x g, 10 min. An aliquot of the supernatant was either used for immunoprecipitation or loaded directly onto an SDS-Polyacrylamide gel.

2.3.10. RNA extractions

Total nucleic acids were extracted by a modification of the method described by Leaver and Ingle (1971). All operations were carried out at 4°C in sterile conditions. Tissue was homogenized with a pestle and mortar or with a MSE "Atomix" homogenizer at full speed for 1-2 min in 2 volumes of freshly prepared RNA grinding buffer. An equal volume of phenol was added and the solution poured into a centrifuge tube and mixed thoroughly with a whirlimixer. After centrifugation at 2,000 x g for 10 min, the aqueous phase was carefully removed and reextracted once with phenol-chloroform and once with chloroform. Nucleic acids were precipitated with two volumes of cold ethanol and stored at -20°C overnight. The precipitate was collected by centrifugation as before, washed twice in 80% ethanol and dried in a vacuum desiccator.

The dry pellet was resuspended in sterile water and RNA precipitated by the addition of potassium acetate pH 6 to 2 M final concentration and stored at 4°C overnight. RNA was pelleted by centrifugation (9000 x g, 10 min), washed twice with 80% ethanol and resuspended in water

to a concentration of 3 mg/ml.

RNA concentration was estimated by measuring the OD at 260 nm of a 1:200 dilution in water. It was assumed that 1 OD unit at 260 nm was equivalent to 30 ug/ml of RNA. An indication of purity of the RNA was obtained by measuring the ratio of absorbances 260/280, which was generally 1.95. RNA was stored at -80°C. A typical preparation yielded about 70 ug/g of tissue (fresh weight).

Poly A⁺ containing RNA was recovered from total RNA by binding to and elution from an oligo dT cellulose column according to Efstratiadis and Kafatos (1976). All steps were performed at room temperature. Oligo dT cellulose (1g) was resuspended in sterile water and the suspension packed into a 5 ml disposable plastic syringe with glass wool at the bottom, forming a 1 x 1 cm bed. The column was washed with binding buffer (0.5 M NaCl, 0.01 M Tris, pH 7.5) until the absorbance at 260 was 0 (about 10 mls). Total RNA (usually 15 mg, 3 mg/ml) was made 0.5 M with 5 M NaCl and 0.01 M with 1 M Tris pH 7.5. The RNA was then heated at 65°C for 5 min, rapidly cooled on ice and loaded onto the column. The column was washed with 10 mls of binding buffer and the bound material eluted with 4 mls of 0.01 M Tris pH 7.5. At this stage, the fraction eluted, poly A⁺ enriched RNA, contained ca. 10% of ribosomal RNA as judged by non-denaturing agarose gel electrophoresis. To obtain poly A⁺ RNA, the enriched fraction was reapplied to the column and eluted as

before. The flow rate was kept at 0.25 ml/min throughout the process. After the second elution, two volumes of cold ethanol were added to the eluate and the mixture stored at -20°C overnight. The Poly A+ RNA was collected by centrifugation, washed twice with 80% ethanol and made 0.25 mg/ml in sterile water. Typically about 1% of total RNA was recovered as poly A+ RNA.

2.3.11 Protein synthesis in vitro

The wheat germ cell-free system of Roberts and Patterson (1973) was prepared and used for in vitro translation of RNA in the presence of ^{35}S -methionine or a mixture of tritiated amino acids. The extraction was performed at 4°C . 6g of non-roasted wheat germ and an equal amount of sterile powdered glass were vigorously ground in a mortar and pestle to a fine powder. Two volumes (15 ml) of WG buffer were added and grinding continued until a paste formed. The mixture was transferred to a corex tube and centrifuged at $30,000 \times g$ for 15 min. The supernatant was carefully removed and applied to a column (30 x 1.5 cm) of Bio Gel P-6DG (Bio Rad, Laboratories) equilibrated in WG buffer. The flow rate was 3 ml/min. Fractions (1 ml) were collected and the OD at 260 nm determined of a 1:200 dilution in water of each fraction. Those fractions with OD at 260 equal or higher than 0.5 were pooled and centrifuged as before. The supernatant was quickly frozen in acetone-dry ice and stored in small aliquots (50 μl) at -80°C . The translation mixtures contained 0.24 mg/ml of total RNA,

2.5 ul of wheat germ, 28 mM Hepes pH 7.6, 120 mM potassium acetate, 2.25 mM magnesium acetate, 0.25 mM spermidine, 1 mM ATP, 50 mM GTP, 8 mM creatinine phosphate, 5 ug of creatine phosphokinase, 2 mM DTT and 25 uM of each amino acid except the labelled ones in a final volume of 25 ul. Typically, 2-4 uCi of tritiated aminoacids or 10-15 uCi of ^{35}S -methionine were included in the mixtures. The reactions were optimized for potassium and magnesium. The incubations were for 90 min at 25°C. Incorporated radioactivity was determined by a modification of the filter paper method of Mans and Novelli (1961). 2.5 ul aliquots were spotted onto 1 cm squares of 3 MM filter paper. The filters were dried at room temperature for 15 min and then incubated succesively for 30 min in cold 10% trichloroacetic acid (TCA), 15 min in boiling 5% TCA and 5 min in cold 5% TCA. Filters were then transferred to a mixture of ethanol-ether (1:1, v/v) at 37°C for 15 min and to ether at 37°C for 10 min. Filters were then dried at room temperature and counted in 5 mls of 0.4% butyl-PBD in toluene.

For processing experiments, the in vitro translation mixture was supplemented with microsomal membranes from bean cotyledons, prepared and used as decribed by Davies and Delmer (1979). In vitro translation products were analyzed on 12% SDS polyacrylamide gels and gels prepared for fluorography as described before.



2.3.11.1 Partial proteolytic digest

A partial proteolytic digest was performed on the in vitro synthesised chitinase as described by Cleveland (1983). After the translation reactions were run on SDS-PAGE, the gel was dried and exposed to pre-flashed X-ray film.

In vitro chitinase was localized in the dry gel and the band excised and digested with protease V8 or chymiotrypsin following the method of Cleveland (1983). After digestion, the products were analysed by SDS-PAGE and fluorography. The same procedure was followed to digest in vivo synthesised chitinase. Purified chitinase was employed in this case. The products were analysed by SDS-PAGE.

2.3.12 Immunoprecipitation

In vitro synthesized chitinase was immunoprecipitated according to Anderson and Blobel (1983). In vitro translation mixtures (50 μ l) were diluted with four volumes of IPDB. 5 μ l of chitinase antiserum were added and the mixture centrifuged (12,000 \times g, 5 min). The supernatant was transferred to a fresh eppendorf tube and incubated overnight at 4°C. Next day about 5 mg of protein A-sepharose swollen in PBS were added and the mixture left at room temperature for 2 h with occasional gentle whirlmixing. The sepharose beads were then pelleted (12,000 \times g, 5 min) and after removing the supernatant, washed 3 times with 1 ml of IPWB. The beads were given a final wash in the same buffer but without

detergents. Finally, the beads were resuspended in sample buffer (62.5 mM tris-HCl ^{pH} ~~7.4~~ 6.8, 2.5% SDS, 200 mM DTT and 10.5% Ficoll) and boiled for 5 min. The beads were pelleted as before and the supernatant loaded onto an SDS-polyacrylamide gel. An identical procedure was employed for immunoprecipitation of in vivo synthesized chitinase except that 15 ul of antiserum and 100 ul of crude homogenate were used.

2.2.14 Complementary DNA

2.2.14.1 Synthesis

The complementary DNA (cDNA) was prepared by D. Baulcombe (PBI, Cambridge) and P. Kelly (Bioscot, Edinburgh) according to the method of Baulcombe and Key (1980). Using poly A⁺ RNA from ethylene treated tissue as a template, cDNA was synthesized by using reverse transcriptase. The RNA was hydrolyzed with alkali and the single stranded DNA converted into double stranded (ds) with E. coli DNA polymerase I. This reaction produced a "hairpin loop" which was removed with S1 nuclease. Using calf thymus terminal deoxynucleotide transferase (TDT), a homopolymer tail of a specific deoxynucleotide (dCTP) was added at both 3' terminus of the ds cDNA, in order to obtain 'sticky ends' of the molecule. Sticky ends were also produced with TDT at the Pst 1 site of pBR322, but using the complementary deoxynucleotide to that used for the cDNA (dGTP). The ds cDNA and the vector were annealed and the hybrid vector resultant used for transformation into E. coli LE

2.2.14.2 Screening

The presence of a DNA insert at the Pst 1 site of pBR322 inactivates the ampicillin resistance gene but leaves the tetracycline resistance gene intact (Bolivar et al . 1977). It is possible, therefore, to identify colonies containing recombinant DNA by the ampicillin-sensitive, tetracycline-resistant phenotype. The selected clones were analyzed by the mini-lysate method of Barnes (1977). Bacteria containing recombinant plasmids were grown on LB plates. About 1 mm of cells were picked up from each streak or single colony with a toothpick and dissolved in 70 ul of TPE. 30 ul of 'hot SDS' were added with mixing. After heating at 65°C for 0.5 h, the lysate was sheared by passage through a 21-gauge needle, using a 1 ml plastic syringe. 10 ul of this solution were run on an 1.2% agarose gel in TPE.

2.2.14.3 Transfer of DNA onto nitrocellulose filters

Recombinant plasmid DNA was transferred according to Southern (1975). Gels containing DNA were incubated after electrophoresis, in Southern denaturing solution for 25 min and in Southern neutralizing solution 2 x 10 min. Gels were placed on a wick made of three thicknesses of 3 MM paper soaked in 20X SSC. The wick was supported on a glass plate between two tanks each containing 20X SSC. A piece of nitrocellulose (0.45 um, pore size) was cut to fit the gel, wetted and equilibrated with 20X SSC. The filter was gently lowered

onto the gel and covered with six sheets of 3 MM paper soaked in 20X SSC, followed by a 6 cm stack of paper towels and a 1 kg weight. Air bubbles were avoided in all steps. Transfer was allowed to proceed for at least 16 h. The nitrocellulose was dried at room temperature and baked at 80°C for 2-3 h in vacuo .

2.2.14.4 Hybridization

Southern blots were hybridized in polyethylene bags at least for 2 h at 65°C in prehybridization buffer. Hybridization was carried out in fresh prehybridization buffer containing the radioactive probe which was end labelled poly A+ RNA (see below). In order to identify clones containing cDNA's to abundant mRNA's, the filters were hybridized to labelled poly A+ RNA from ethylene treated tissue. Positive clones were picked up and hybridized to poly A+ RNA from both control and ethylene treated tissue. Clones responding specifically to RNA from ethylene treated tissue were selected for further analysis.

2.2.14.5 Hybrid release translation

Plasmid DNA was linearized by digestion with Eco R1, separated by electrophoresis in a 1.2% agarose gel and blotted onto nitrocellulose membranes as described before. After drying and baking, the filters were prehybridized for two days at 65°C in 0.4 M sodium acetate pH 6, 1 mM EDTA (Miller et al . 1980). The filters were then hybridized in the same buffer plus 400

~~leaves~~ ^{ZONES}

ug of total RNA from ethylene treated ~~leaves~~ ^{ZONES} per filter in a final volume of 100 ul at 60°C for 4 h. After removing the supernatant, the filters were washed ten times with 1 ml of 0.5X SSC, 0.5% SDS at 65°C and once in 2 mM EDTA. The selected RNA was eluted by boiling for 2 min in 300 ul of 1mM EDTA and then quick frozen in ethanol/dry ice. The supernatant was removed, made 0.2 M sodium acetate, 10 ug of wheat germ transfer RNA added and RNA precipitated with three volumes of ethanol at -20°C. The RNA was pelleted, washed twice in 70 % ethanol and used for in vitro translation in the wheat germ system as described before. The translation components were added directly to the RNA. After translation, each mixture was subjected to immunoprecipitation as described before and the products run on an SDS-polyacrylamide gel.

2.2.15 Plasmid extraction

2.2.15.1 Growth conditions

E. coli derived plasmid pBR322 (Bolivar et al. 1977) and recombinant plasmids were maintained in HB 101 cells. Colonies were grown at 37°C overnight on LB plates containing, when appropriate, a permissible antibiotic (tetracycline, 10 ug/ml), and stored at 4°C (Maniatis et al. 1982). For long-term storage, an aliquot of the liquid cultures (1 ml) was stored at -80°C in the presence of 15% glycerol (Maniatis et al. 1982). When required, bacteria were grown in LB medium liquid cultures (usually 400 mls) on an orbital shaker

(New Brunswick) at 300 rpm until an OD at 600 nm of 1 was reached. Chloramphenicol was added to a final concentration of 100 ug/ml and the culture 'amplified' overnight.

2.2.15.2 Large scale preparation

Plasmid DNA was prepared from a single bacterial colony by the alkaline method of Birnboim (1983). Cells were collected by centrifugation (6000 x g, 10 min) and washed once with distilled water. The cell pellet was resuspended in lysis buffer and incubated on ice for 0.5 h. Alkaline buffer and high salt buffer were added to the suspension and incubation continued for another 0.5 h. The insoluble material was removed by centrifugation and two volumes of ethanol added to the supernatant. After centrifugation the pellet was kept and resuspended in CT, the solution was heated at 60°C for 15 min and ammonium acetate added to a final concentration of 3.75 M. The precipitate was removed as before and nucleic acids ethanol precipitated. Nucleic acids were then pelleted by centrifugation and plasmid DNA purified by one cycle of cesium chloride gradient centrifugation for 48-50 h at 40,000 x g. Plasmid DNA was washed three times with two volumes of water saturated-butanol and ethanol precipitated at -20°C. DNA was resuspended in CT at a concentration of 0.5 mg/ml. An aliquot was run on an agarose gel to check the purity of the preparation. Typically from 2-3 mg of plasmid DNA were obtained per 1l of medium.

2.2.15.3 Plasmid DNA mini preparation

In essence an identical procedure to the one outlined above and based on Birboimⁿ (1983) was followed, except that the volumes were scaled down to 1 ml samples and there was no caesium chloride centrifugation. The crude preparation was digested with a restriction enzyme and loaded on a 1.2% agarose gel.

2.2.16 Restriction Enzyme digestion of DNA

Recombinant plasmid DNA was digested at a concentration of 0.05-0.1 ug/ul in volumes of 10-20 ul in 1X Restriction enzyme buffer with 2-3 units of enzyme per ug of DNA. Digestion was performed at the recommended temperature for 1-3 h and terminated by heating at 65°C for 10 min or by phenol extraction.

2.2.17 DNA gel electrophoresis

Horizontal gels (1.2%) were run in TAE or TPE buffer. 3 ul of DEF were added to each sample before the run. Electrophoresis was carried out at 4 V/cm until the dye had traversed 3/4 of the gel. Gels were stained with ethidium bromide (1-5 ug/ml in the electrophoresis buffer) for 0.5 h, destained in two changes of water for 1 h and photographed under UV light.

When necessary, DNA was run in low gelling temperature agarose in the same conditions as above except that the run was at 4°C. DNA was recovered from the gels (Wieslander, 1979) by excising the DNA bands after electrophoresis. The agarose was melted at 65°C for 15 min and the DNA phenol extracted and ethanol

precipitated.

2.2.18 RNA electrophoresis and blotting on to nitrocellulose

Total RNA was electrophoresed on 1.2% agarose gels containing formaldehyde as described by Maniatis et al . (1982). After electrophoresis, the RNA was immediately transferred on to nitrocellulose as described by Thomas (1980).

After the transfer, the filters were baked for two hours under vacuum and prepared for hybridization.

2.2.19 Preparation of radioactively labelled probes

2.2.19.1 DNA

Nick translation of DNA (Rigby et al . 1977) was carried out as described by Maniatis et al . (1982) in a final volume of 50 ul. The reaction mix contained 1X NT buffer, 1 nm of each dNTP except dCTP, 0.5 ug of DNA, 5 ug of DNase 1.5 units of E. coli DNA pol I and 10-20 uCi of 32 P-dCTP. After 1 h at 14°C, the reaction was stopped by adding 5 ul of 0.5 M EDTA and 150 ul of STE. Unincorporated radioactivity was separated by chromatography in Sephadex G-50 in STE (Maniatis et al . 1982). The probe was used at ca. 1,000,000 cpm/ml of hybridization buffer.

2.2.19.2 RNA

Poly A+ RNA was kinase labelled by a modification of the method of Maniatis et al . (1982). RNA was first heated at 100°C for 3 min in 20 ul of 1X kinase buffer and cooled on ice. The kinase labelling reaction

contained the RNA solution, 6 units of T4 polynucleotide kinase and 15-25 uCi of ^{32}P -ATP. The mixture was incubated at 37°C for 0.5 h and stopped with 10 ul of 0.5 M EDTA. Unincorporated radioactivity was separated as described above.

2.2.20 Screening of a bean genomic library with an oligonucleotide probe

A Phaseolus vulgaris genomic library was obtained from Agrigenetics. It was constructed by cloning 15-20 Kbp fragments of Mbo I-digested P. vulgaris total DNA into the lambda vector charon 34 (Loenen and Blattner, 1983).

2.2.20.1 Growth of plating cells

E. coli DH 1 was grown on LB plates at 37°C overnight. A single colony was used to inoculate 50 mls of LB medium, supplemented with 0.2% maltose and grown with shaking until stationary phase. Cells were harvested by centrifugation at 2500 rpm for 10 min and resuspended in 0.01 M magnesium sulphate at an OD at 600 nm of 2 (ca. 1.5×10^8 cells/ml).

2.2.20.2 Plating bacteriophage

Serial dilutions of phage in 0.01 M magnesium sulphate were absorbed to 0.1 ml of plating bacteria in sterile glass tubes for 20 min at 37°C. 2.5 ml of top agarose (at 50°C) were added and the mixture plated onto dry 9 cm LB plates. The plates were incubated for 8-14 h at 37°C. The library was plated at a density of about 5000 plaque forming units (pfu) per petri dish.

2.2.20.3 Phage transfer onto nitrocellulose

Plaque lifts were performed according to Benton and Davis (1979) as described by Maniatis et al . (1982). Plates were overlaid with precut circles of nitrocellulose (0.45 μ m, pore size). Orientation marks were provided by marking the filters with black ink. The nitrocellulose was left in contact with the plates for 45 seconds, then peeled off and laid 'plaques' side up on a piece of 3 MM paper soaked in Southern denaturing solution. After 2 min, the filter was transferred to a second piece of 3 MM paper soaked in Southern neutralizing solution for a further 2 min and finally to another piece of 3 MM paper soaked in 0.5 M Tris pH 7.5, 1.5 M NaCl for 2 min. The filters were dried onto 3 MM paper and baked at 80°C for 2-3 h under vacuum.

2.2.20.4 Oligonucleotide hybridization

The filters with the DNA bound were prewashed in 3X SSC/0.1% SDS at 65°C for 16-20 h. The prehybridization was done in 6X SSC, 1X Denhardt's solution or 0.1 mg/ml heparin, 0.5% SDS, 0.05 sodium pyrophosphate and 100 μ g/ml sheared and denatured salmon sperm DNA at 37°C for 2-3 h. The hybridization was carried out in the same buffer plus the labelled oligonucleotides at an specific activity of 1.4×10^6 cpm/ μ g of DNA at 1,000,000 cpm/ml of hybridization buffer. The minimal melting temperature (T_m) for hybridization was calculated by using the empirical formula of Suggs et al . (1981)

$$T_m = 2C + 4G$$

where C= number of dA-dT base pairs and

G= number of dG-dC base pairs.

to be 66°C. Hybridization and washing of the filters were performed at different stringencies in order to optimize the conditions and reduce the nonspecific hybridization (see chapter 6). After washing and drying, the filters were exposed to pre-flashed X-ray film at -70°C.

2.2.20.5 Oligonucleotide probe

A partial amino acid sequence of the N-terminus of pure chitinase was determined by L. Fothergill (Aberdeen) by the Edman degradation method. Based on this information, two mixtures of oligonucleotides were synthesised by S. Minter (Manchester). The mixtures were used for screening the bean genomic library as described in the chapter 6.

2.2.20.6 Oligonucleotide kinase labelling

The oligonucleotides were 5' end labelled following the method of Maniatis et al . (1982), using ^{32}P -ATP and T4 polynucleotide kinase. The reaction mix contained per 15 ng of oligonucleotide, 1 X kinase buffer, 15 uCi of ^{32}P -ATP and 3 units of T4 kinase in a final volume of 20 ul. After 1 h at 37°C, the reaction was stopped by adding 5 ul of 0.5 M EDTA and heating at 70°C for 10 min. Unincorporated radioactivity was separated by chromatography on Sephadex G-25 as described by Maniatis et al . (1982).

CHAPTER 3 ANALYSIS OF THE IN VITRO TRANSLATION PRODUCTS OF BEAN ABSCISSION ZONES

3.1 Rationale

A cell-free protein synthesizing system is the obvious choice as a first approach to identifying mRNA molecules and studying their properties and the properties of the proteins they code for. The wheat germ lysate (Marcus and Duddock, 1981, Roberts and Patterson, 1978), a system currently used to this end, has been shown to translate primarily cytoplasmic mRNA from plant tissue (Bottomley et al . 1976). The products can then be analyzed on SDS-PAGE. For these reasons, the wheat germ system was chosen in this study. Experiments carried out in this laboratory have shown that the products obtained programming the rabbit reticulocyte lysate with RNA from abscission zones are substantially similar to those obtained with the wheat germ system.

3.2 Isolation of RNA from bean tissue and in vitro translation

RNA to be used for in vitro translation was obtained from bean tissue by a slight modification of the method of Leaver and Ingle (1971). Modifications included homogenization and phenol extraction in alkaline rather than neutral buffer to inhibit ribonuclease activity and to allow partitioning of mRNA into the aqueous phase by preventing the binding of the 3' poly A+ tail of mRNA's to denatured protein (Brawerman et al . 1972, Taylor, 1979). As total nucleic acids were extracted by this

procedure, an additional sodium acetate precipitation step was included to separate the RNA from DNA and carbohydrates (Haffner et al . 1978). The RNA samples obtained for translation consisted largely of ribosomal RNA (rRNA) as revealed by electrophoresis in non-denaturing conditions. A typical preparation yielded 70 ug of total RNA per g of abscission zone tissue.

Loss of secondary structure has been found to improve translation efficiency and to result in a more accurate reflection of the relative abundances of mRNA's (Payvar and Schimke, 1979; Mortensen et al . 1984). Prior to in vitro translation, aggregates of RNA and RNA secondary structure were disrupted by heating to 70°C for 5 min.

The wheat germ system was adapted from that of Roberts and Patterson (1973) as modified by Anderson et al . (1983). In this system, incorporation of tritiated amino acids or ^{35}S -methionine into TCA-precipitable material was found to be completely dependent on exogenous mRNA. The wheat germ system was characterized using total ~~bean~~ ^{from ABS-ZONES} RNA to direct protein synthesis.

3.3 Optimizing the conditions for incorporation of amino acids into protein

It is well established that the efficiency of translation of different mRNA's may vary considerably depending on a variety of conditions (Lodish, 1974). Cations such as potassium and magnesium and the polyamines spermidine and spermine are particularly

important in determining total amino acid incorporation (Roberts and Patterson, 1973; Hunter et al . 1977). The concentration of RNA is also important as high levels of RNA may strongly or completely inhibit incorporation (Longa re and Rulter, 1977; Sononstein and Brawerman, 1976).

For all these reasons, it was necessary to optimize the cell-free system for total incorporation of amino acids into protein. The cations magnessium and potassium were found to affect translation. Low concentrations of either allowed only a poor rate of translation whereas an increase produced a concomitant increase in incorporation. Magnesium was optimised in the presence of spermidine as the presence of polyamines lowers the requirement for magnesium (Igarashi et al . 1975, 1978). The optimal concentrations were found to be 130 mM for potassium and 2.5 mM for magnessium (figure 2). Therefore these concentrations were routinely used in this study.

The concentration of RNA had a profound effect on the incorporation of amino acids into protein. Incorporation increased with increasing RNA up to 6-8 ug, decreasing thereafter. With a single preparation of RNA the molecular weight distribution of translation products was independent of the amount of RNA included in the system.

As has generally been found with the wheat germ system (Roberts and Patterson, 1973; Marcus and Duddock, 1983),

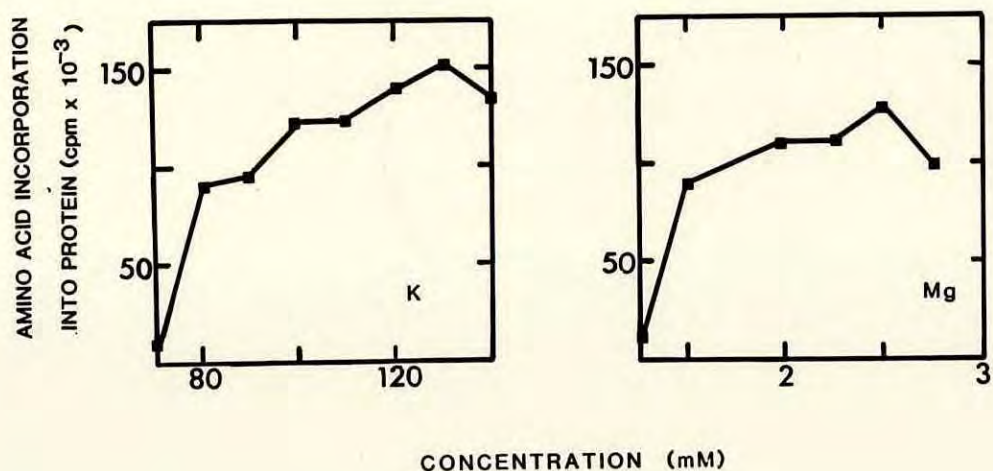


Figure 2 Effect of the concentration of potassium and magnesium on the incorporation of labelled amino acids into protein in the wheat germ in vitro translation system programmed with RNA from ethylene-treated abscission zones

RNA extracted from ethylene-treated abscission zones was translated in the wheat germ lysate and the incorporation of tritiated amino acids into protein determined. Different concentrations of magnesium and potassium were tested. The concentration of magnesium was 2.5 mM when potassium was being assayed. Potassium was kept at 120 mM when magnesium was being optimized.

increases in potassium and magnesium led to a reduction in amino acid incorporation into proteins. No differences were observed in the relative amounts of in vitro translation products in the range of concentrations used in this study for both cations.

As wheat germ contains a whole range of factors potentially influencing translation (Marcus et al. 1974; Shafinitz, 1977), specifically as well as non-specifically, the system was optimized for wheat germ. No relative differences were detected on SDS-PAGE in the pattern of in vitro translation products using different amounts of wheat germ. An optimal concentration of 2.5 ul per 25 ul incubation mix was used in the final conditions for incorporation.

The time course for amino acid incorporation was linear up to 60 min; after an initial lag period of about 10 min and was essentially complete by 90 min. Two different labels, tritiated amino acids or ³⁵S-methionine, were used with the wheat germ. Some in vitro translation products were enhanced depending on the label used. ³⁵S-methionine was used in immunoprecipitation experiments of in vitro translation products to visualize the radioactive proteins in a shorter time.

3.4 Analysis of the translation products of the wheat germ directed by bean total RNA

RNA isolated from bean tissue directed the synthesis de novo of proteins as judged by the incorporation of

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³⁵S-methionine and tritiated amino acids into material precipitable by TCA. The labelled translation products were fractionated by SDS-PAGE and visualized by fluorography. The incorporation of the label into TCA-precipitable material represented the synthesis of discrete polypeptides of high molecular weight (see figures 3, 4 and 5). The fact that high MW products can be detected by in vitro translation, suggested that the RNA preparation was not extensively degraded during isolation. As the profile of products directed by total RNA and poly A⁺ RNA was very similar, total RNA was routinely used in this study for in vitro translation experiments. In addition this approach can eliminate the possibility that certain mRNA species were selectively lost during isolation; for instance they might lack of poly A⁺ tail and so not adhere to the oligo dT cellulose.

3.5 Comparison of the translatable mRNA population from abscission zones, stems, petioles and leaves during ethylene treatment

Total RNA samples were prepared from abscission zones explants that had been exposed to 50 ppm ethylene for up to 72 h. The abscission zone region was divided into 3 x 5 mm segments, those containing the abscission zone itself and the stem and petiole segments immediately proximal and distal to it. These RNA extracts were used to programme the wheat germ lysate. In parallel experiments, bean leaves still attached to the stem were

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incubated in 50 ppm ethylene and RNA prepared from them. RNA preparations from abscission zones, leaves, petioles or stems directed the synthesis of a large variety of proteins when translated in the wheat germ system. Clear differences in the profile of translation products of each tissue were apparent after various periods in ethylene (cf. figures 3 and 4). There were three major products, with a mw of 42, 32 and 17 kD, which accumulated substantially on ethylene treatment in abscission zones and petioles (figure 3). In stems, only the 17 kD product was strongly induced after 72 h of ethylene treatment, although there were some other minor polypeptides which showed an increase or a decrease on ethylene induction (figure 3). The 42 and 32 kD products were present in stems but not in the same relative amounts as in zones or petioles. No translation products were identified by this method of analysis, which were solely found in abscission zones.

In the case of leaves, the pattern of in vitro translation products was clearly different from that of other tissues (figure 4). The 42 kD and 32 kD products were detected and they accumulated on ethylene treatment. They seemed to be less abundant in leaves than in abscission zones or petioles and were not detected in the absence of ethylene.. The prominent products in leaves were one of 20 kD and a group of products of 30 kD. The 20 kD product was present all the time, either in the presence or absence of ethylene

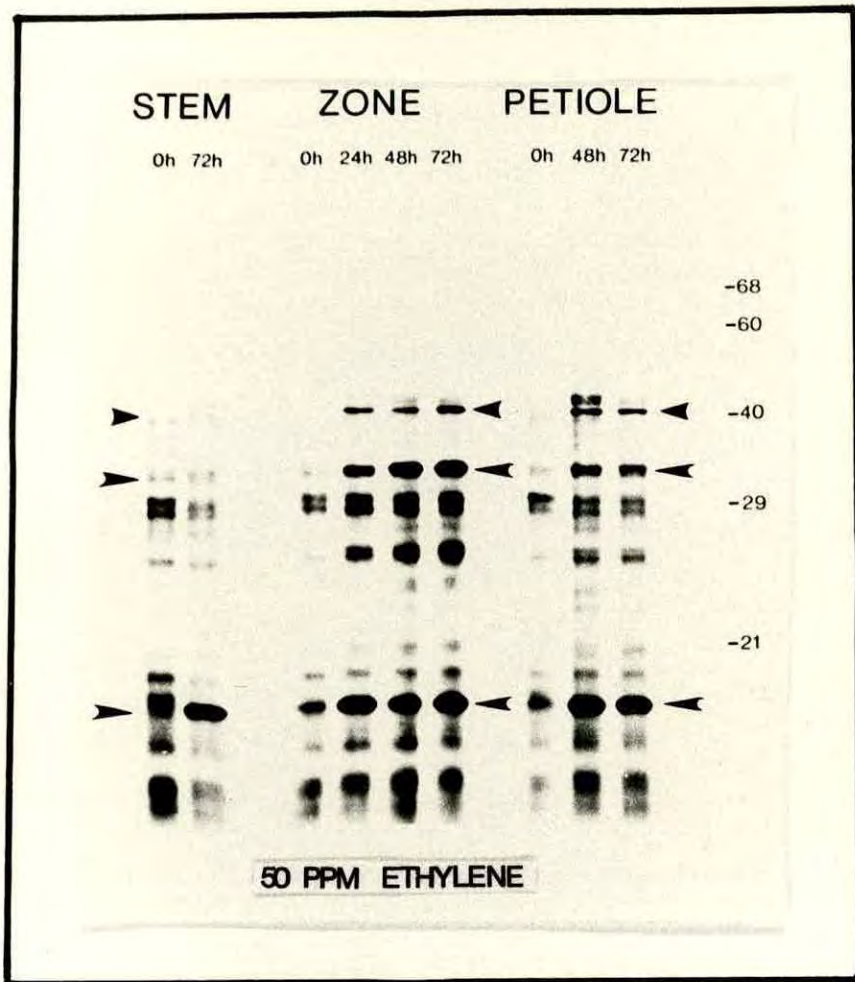


Figure 3 Comparison of in vitro translation products of stem, petiole and abscission zone.

Bean explants were treated with ethylene for different periods of time. The different organs were then excised and total RNA extracted from them. The RNA was translated in vitro and the products analysed by SDS-PAGE and fluorography. The numbers at the top indicate the incubation time in ethylene. The numbers on the right correspond to molecular weight markers (in kilodaltons). The arrows indicate the three major in vitro translation products which accumulate during ethylene treatment. About 300,000 cpm were loaded per track.

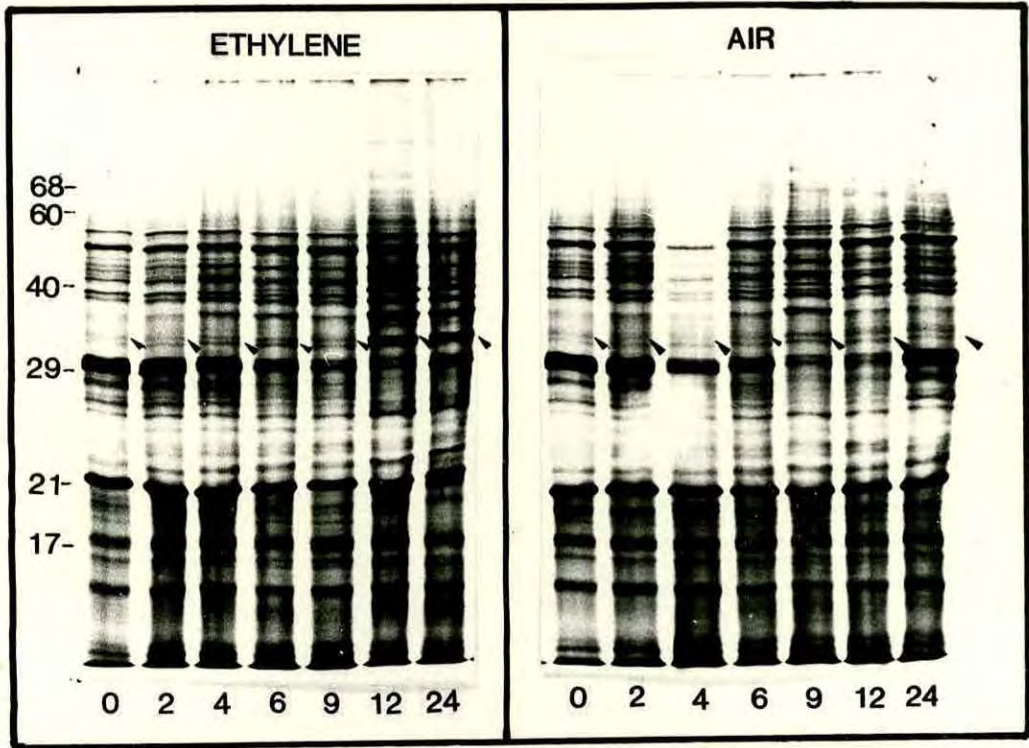


Figure 4 Changes in the mRNA translation products from leaves during ethylene or air treatment.

Bean seedlings were incubated in ethylene or air for different periods of time. The leaves were excised and RNA was then extracted from them and translated in the wheat germ lysate. The products were analysed by SDS-PAGE and fluorography. The numbers at the bottom indicate the time of incubation in hours. The numbers on the left correspond to molecular weight indicators (in kilodaltons). The arrows indicate the position of the 32,000 in vitro translation product. About 400,000 cpm were loaded per track.

(figure 4). On the other hand the 30 kD group of proteins declined after 9 h of incubation either in ethylene or in air. The 17 kD product did not seem to be present in leaves.

3.6 Analysis of the changes in the translatable mRNA population in the abscission zone during ethylene induction

The time course of the changes in translatable mRNA in abscission were examined in more detail (figure 5). Three major products of mw 42 kD, 32 kD and 17 kD accumulated during ethylene treatment. The 42 and 32 kD products appeared to be synthesized de novo as they could not be detected at the beginning of the ethylene treatment. The 42 kD product was just detectable by 6 h and increased to a maximum by about 20 h. The 32 kD product had visibly increased by 6 h reaching a maximum at 20 h. The 17 kD product was present at the beginning of the treatment and reached its peak by 6 h. There was a minor band at 27 kD which also accumulated in abscission zones. This product increased by 6 h of ethylene treatment and reached a maximum by 20 h.

Were these products involved in abscission? How were they regulated by ethylene? What were the in vivo products? It was known that a number of hydrolytic enzymes showed an increase in activity during abscission (Addicot, 1982; Sexton and Roberts, 1982) and that ethylene stimulated abscission dramatically (Abeles, 1973; Jackson and Osborne, 1970). On the other hand, RNA

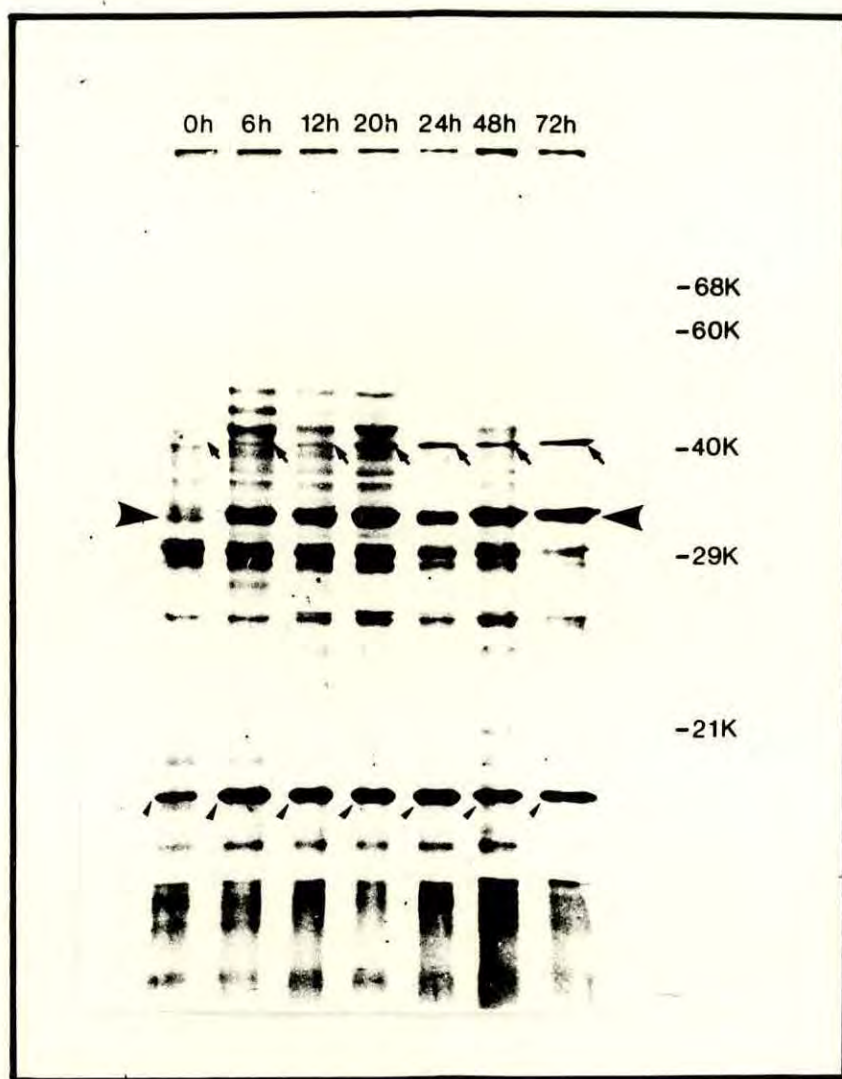


Figure 5 Changes in mRNA translation products from abscission zones during ethylene treatment

Bean explants were incubated with ethylene for different periods of time. The zones were isolated and total RNA extracted from them and translated in vitro. The products were analysed by SDS-PAGE and fluorography. The numbers at the top indicate the time of incubation in ethylene. The numbers on the right correspond to molecular weight markers. The arrows indicate the position of the three major translation products which accumulate on ethylene treatment. About 300,000 cpm were loaded per track.

and protein synthesis inhibitors had been shown to prevent abscission (Abeles, 1969; Goren et al . 1969). It was possible, then, that the three major in vitro translation products induced by ethylene might be related to abscission, though none of these changes seemed exclusive to the abscission zones.

Immunological evidence (Kelly et al . in preparation) has suggested that the 42 kD product corresponded to cellulase, which is the main enzyme involved in abscission (Horton and Osborne, 1967; Sexton et al . 1980).

In 1983 Boller et al . showed chitinase to be an ethylene-regulated enzyme present in bean leaves. The reported mw for chitinase was 30 kD which was sufficiently close to the mw of the 32 kD in vitro translation product to suggest they might be the same. My work consisted from then on in establishing this identity. There are still no identity assigned to the 17 and 27 kD translation products.

3.7 Identification of the 32 kD in vitro translation product as chitinase

After the initial suggestion that the 32 kD product and chitinase were the same, I set out to confirm this identity by using several lines of evidence. Each will be considered in turn.

3.7.1 Processing of the 32 kD product by microsomal membranes

The work of Boller et al . (1983) has shown that

chitinase accumulates during ethylene treatment. On SDS-PAGE, chitinase has an apparent mw of 30 kD. There was a difference of 2 kD between chitinase and the 32 kD product. It was possible, assuming that both products were the same, that the difference was due to some form of postranslational modification. It is well established that proteins can be 'processed' in various ways (Wold, 1979) before they resemble their in vivo counterparts. Modifications such as phosphorylation (Trewavas, 1976), glycosylation (Chrispeels, 1976) or removal of a leader sequence (Blobel and Dobberstein, 1975) are known to occur in proteins.

As a first approach to solving this problem, microsomal membranes from bean cotyledons were included in the translation mixtures to attempt to process ^{the} 32 kD product ~~processed~~. The figure 6 shows the result of such experiment. The 32 kD product was processed to one of 30 kD which is the mw of in vivo chitinase.

3.7.2 Antigenic identity between the 32 kD translation product and chitinase Antibodies are essential in many areas of research. Their specificity provides an unvaluable tool especially for purposes of identification of proteins. For this reason they were used in this study.

Chitinase was purified to homogeneity from abscission zones using the method developed for leaves by Boller

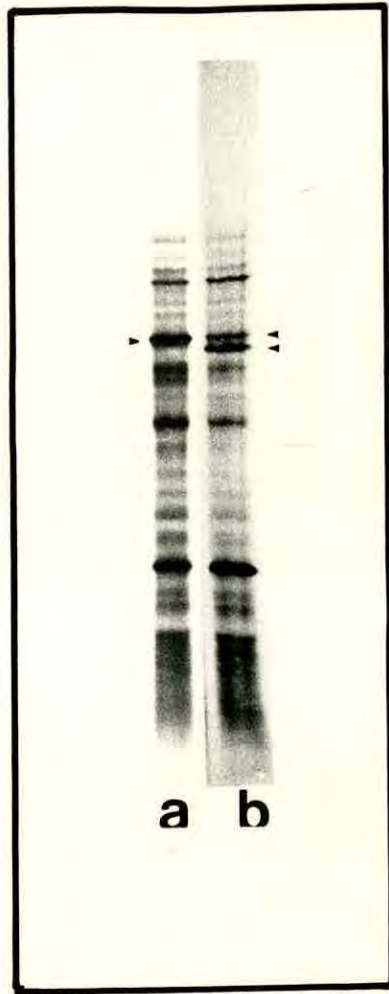


Figure 6 Processing of the 32,000 in vitro translation product by microsomal membranes from bean cotyledons

Total RNA was extracted from abscission zones was translated in vitro in the wheat germ lysate in the presence of microsomal membranes extracted from bean cotyledons. After translation, the products were analysed by SDS-PAGE and fluorography. The figure illustrates total translations products in the absence (A) or presence (B) of microsomal membranes.

et al . (1983). The enzyme was pure as judged by SDS-PAGE (see section 4.2). Antibodies were raised in rabbits against this protein. Immunodiffusion and Immunoelectrophoresis (Clausen, 1971) were carried out to confirm both the homogeneity of the purified enzyme and the specificity of the antiserum. Immunodiffusion experiments showed no reaction between the antiserum and proteins from untreated tissue and a single precipitin line with proteins from treated tissue (figure 7). There was no reaction with preimmune serum. Incubation of the antiserum with purified chitinase resulted in inactivation of the enzyme (Table 1).

Antibody specificity is illustrated in the figure 8 an immunoelectrophoresis experiment, which shows a major single precipitin line obtained by challenging a crude extract of ethylene treated abscission zones separated by electrophoresis with chitinase antiserum. A second small band was also detected in this experiment which was not visible in immunodiffusion. The presence of this band may be due to contamination of the sample used to raise the antibodies with another protein. Alternatively, it may be due to binding of the antibodies to a protein antigenically related to chitinase.

The chitinase antiserum was also used to precipitate chitinase from tissue labelled in vivo with ³⁵S-methionine. The label was applied to abscission zones and the zones treated with ethylene. This experiment

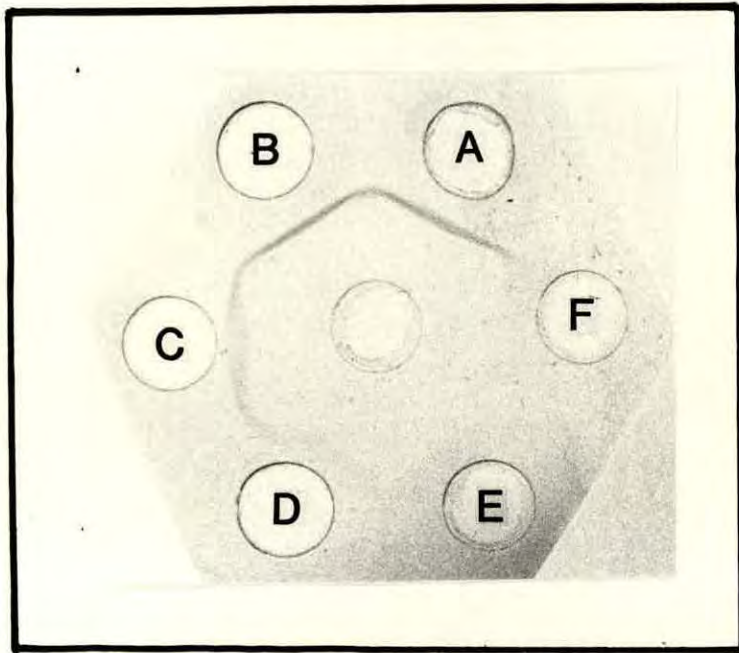


Figure 7 Estimation of the activity of the chitinase antiserum in Ouchterlony plates.

Outer wells of a 1.5% agarose plate in PBS were filled with a constant amount (50 μ l) of progressive dilutions of a crude homogenate from ethylene-treated abscission zones. The centre well was filled with antiserum to chitinase. Diffusion was allowed to proceed for 24 h at 4°C. The plate was then washed, dried, stained with 0.2% coomassie blue and destained. A, crude homogenate undiluted. B, 1:1 dilution. C, 1:2 dilution. D, 1:4 dilution. E, 1:8 dilution. F, crude homogenate undiluted from air-treated abscission zones.

Table 1 Effect of chitinase antiserum and preimmune serum on enzyme activity

Chitinase (6.5 units) was incubated with or without chitinase antiserum in PBS for 2 h at 37°C and overnight at 4°C in a final volume of 0.5 ml. The complex was then pelleted (12,000 x g, 10 min) and 0.25 ml of the supernatant assayed radiometrically.

| Enzyme (units) | antiserum (ul) | enzyme activity (nmol of diacetyl chitobiose released) | inhibition (%) |
|-------------------|-------------------|--|-------------------|
| 6.5 | 10 | 0.87 | 87 |
| 6.5 | 20 | 0.74 | 89 |
| 6.5 | 50 | 0.8 | 87 |
| 6.5 | 50 (preimmune) | 6.2 | 3 |
| 6.5 | 0 | 6.4 | 0 |

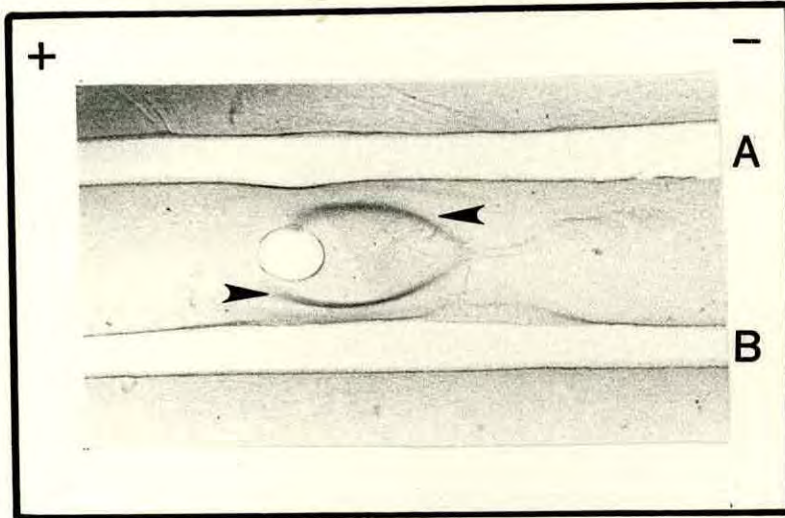


Figure 8 Immunoelectrophoretic demonstration of antibody specificity

The center well of a 1% agarose plate in 0.05 M barbital buffer pH 8.2, was filled with a crude homogenate of ethylene-treated abscission zones. The homogenate was electrophoresed in the same buffer at 4 v/cm for 2 h. The troughs were then removed and antiserum against chitinase was placed on each. Immunodiffusion was allowed to proceed for 24 h at 4°C. The plate was then processed as for the Ouchterlony plate. A and B correspond to two different batches of antiserum. The antiserum in A was used for all the immunoprecipitation experiments. The arrows show precipitin lines. The plus and minus symbols refer to the position of the electrodes during electrophoresis.

would therefore be measuring protein synthesis in vivo. A crude homogenate was prepared and incubated with chitinase antiserum. The precipitate was removed and run on SDS-PAGE. A single band of 30 kD which comigrated with purified chitinase was obtained (figure 9). No band was obtained when pre-immune serum was employed for the immunoprecipitation.

3.7.2.1 Immunoprecipitation of the 32 kD in vitro translation product using antibodies to chitinase

After this initial characterization, the antiserum was employed in immunoprecipitation experiments with the in vitro translation mixture. After translation, the mixture was incubated with the antibodies in the presence of protein A-Sepharose and the protein A-Sepharose isolated and washed. Labelled products were removed by boiling and analysed by SDS-PAGE and fluorography. The 32 kD product was immunoprecipitated by the chitinase antiserum (figure 10).

No precipitate was obtained when pre-immune serum was employed or when in vitro translation products from untreated tissue were incubated with the antibodies to chitinase. Traces of the 42 kD product, possibly cellulase, were sometimes found in the immunoprecipitation. It is not clear whether cellulase is immunologically related to chitinase or is just a non-specific contaminant. It is worth mentioning that a 32 kD product has appeared as a contaminant in immunoprecipitation experiments with avocado using

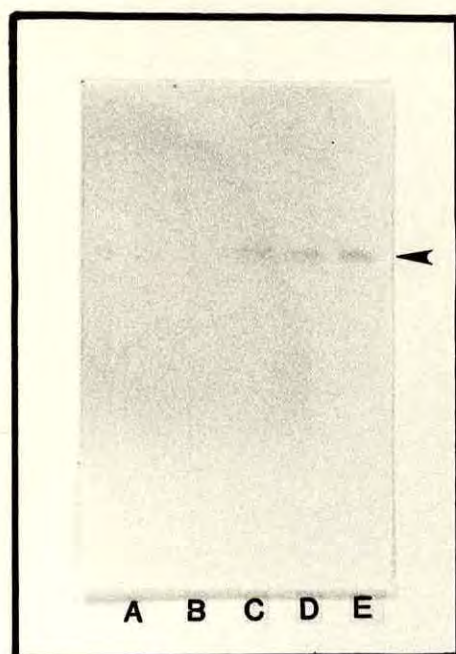


Figure 9 Immunoprecipitation of chitinase from in vivo labelled abscission zones.

Explants were excised from bean seedlings and treated with ethylene for 16 h. Labelled methionine (0.5 uCi) was applied to each zone and the ethylene treatment continued for 12 h. A further 0.5 uCi of methionine was applied per zone and incubated for a final 12 h in ethylene. A crude homogenate was prepared in 0.1 M sodium acetate buffer. Chitinase antibody was added to an aliquot of the supernatant and after incubation at 4°C overnight, protein A-sepharose was added to the mixture. The sepharose beads were washed and pelleted by centrifugation. The fraction bound to the sepharose was released by boiling for 5 min and analysed by SDS-PAGE and fluorography. The arrow indicates the products precipitated. The lanes contain products precipitated by A, 1 ul of chitinase antiserum. B, 2.5 ul. C, 5 ul. D, 7.5 ul. E, 10 ul.

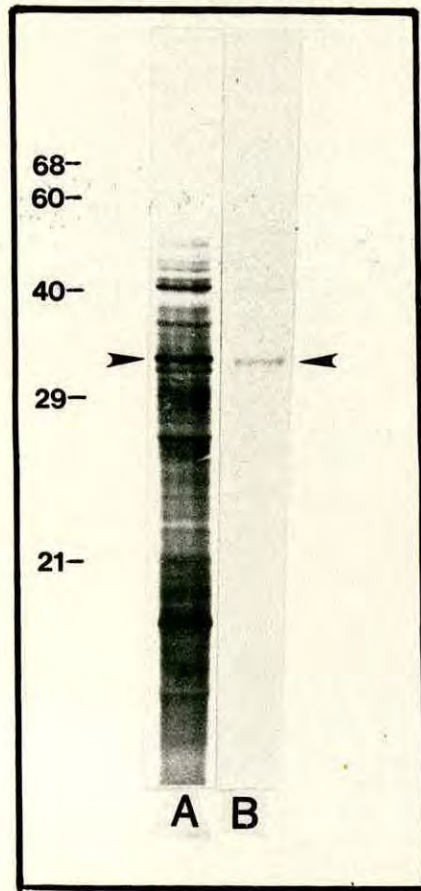


Figure 10 Immunoprecipitation of the 32,000 in vitro translation product by antibodies raised against purified chitinase.

RNA from abscised tissue was translated in vitro in the wheat germ system and the translation mixture treated with antibodies to chitinase (overnight at 4°C). Protein A-Sepharose was then added to the mixture and incubated for 1 h at room temperature. The protein A-Sepharose was washed and the fraction bound to the Sepharose beads was eluted by boiling for 5 min and electrophoresed on a 12% SDS slab gel. The gel was prepared for fluorography, dried and exposed to a pre-flashed X-ray film at -70°C. The lanes contain: A, total translation products. B, products precipitated by chitinase antiserum. The arrows show the position of the 32,000 in vitro translation product.

cellulase antibody (Tucker and Laties, 1984; Christoffersen et al . 1984).

The 32 kD product was also immunoprecipitated when chitinase antiserum was used with translation mixtures programmed with RNA extracted from leaves, stems or petioles (figure 11). No precipitate was obtained when preimmune serum was used or with translation products from non-induced tissue (figure 11).

Chitinase antiserum also precipitated the 32 kD product after processing by the bean microsomal membranes (figure 12). All these experiments suggested that there was an antigenic relationship between the 32 kD product and chitinase.

3.7.3 Affinity chromatography

The purification of chitinase involves an affinity chromatography step, which uses regenerated chitin as substrate (Molano et al . 1977, Boller et al . 1983). If the 32 kD in vitro translation product was a precursor of chitinase, there might be structural similarities to the in vivo enzyme. For this reason it was decided to use the affinity column with the in vitro translation mixture, using the same protocol as that to purify chitinase. After translation, the mixture was run through a column of regenerated chitin. The column was washed and the material bound eluted with acetic acid. This material was then precipitated and washed with ice cold acetone and run on SDS-PAGE. The figure 13 illustrates the result of such experiment. The

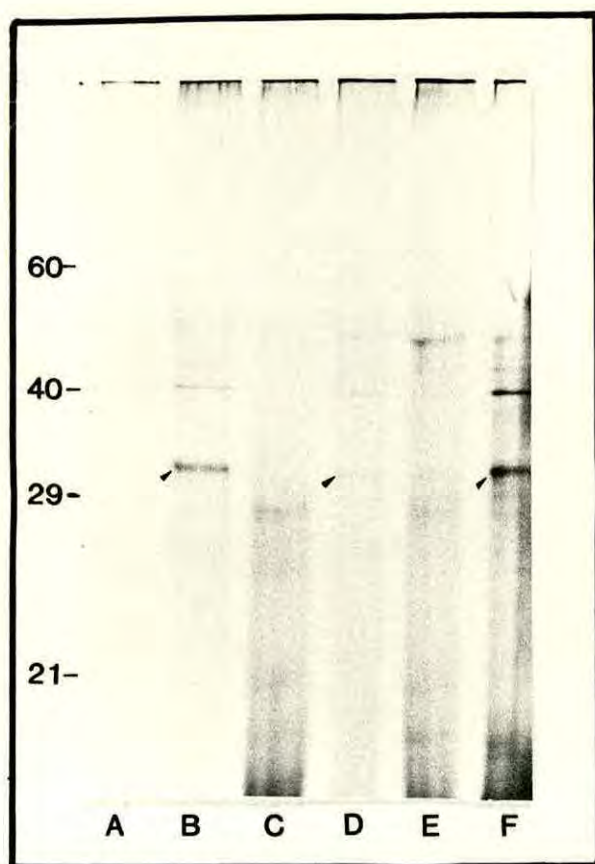


Figure 11 Immunoprecipitation of the 32,000 in vitro translation product by chitinase antibody from translation mixtures programmed with RNA extracted from leaves, petioles and stems.

Bean seedlings were incubated in ethylene for a period of time. Stem, leaves and petioles were excised from the explants and RNA was extracted and used to programme the wheat germ lysate. After translation, chitinase antibody was added to the translation mixtures and after incubation overnight at 4°C, protein A-sepharose was added. The sepharose beads were washed and pelleted by centrifugation. The bound fraction was eluted by boiling for 5 min and analysed by SDS-PAGE and fluorography. The lanes contain translation products precipitated by chitinase antibody from A, leaves before ethylene treatment. B, leaves after 72 h in ethylene. C, stems before ethylene. D, stems after 72 h in ethylene. E, petioles before ethylene treatment. F, petioles after 72 h in ethylene. The arrows indicate the position of the 32,000 in vitro translation product. The numbers correspond to molecular weight indicators in kilodaltons.

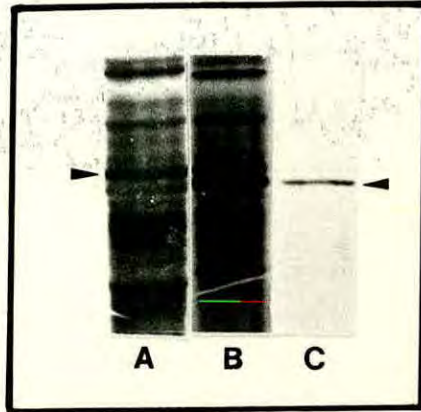


Figure 12 Immunoprecipitation of the 32,000 in vitro translation product after processing by bean microsomal membranes.

RNA from ethylene-treated abscission zones was translated in the wheat germ lysate in the presence of bean microsomal membranes. After translation, chitinase antiserum was added and the mixture incubated at 4°C overnight. Protein A-Sepharose was added and after 1 h at room temperature, the beads were washed and pelleted by centrifugation. The bound products were released by boiling for 5 min and analysed by SDS-PAGE and fluorography. The lanes contain total translation products in the absence (A) or presence (B) of membranes. Lane C contains the products immunoprecipitated by the chitinase antiserum. The arrows indicate the position of the 32,000 in vitro translation product.

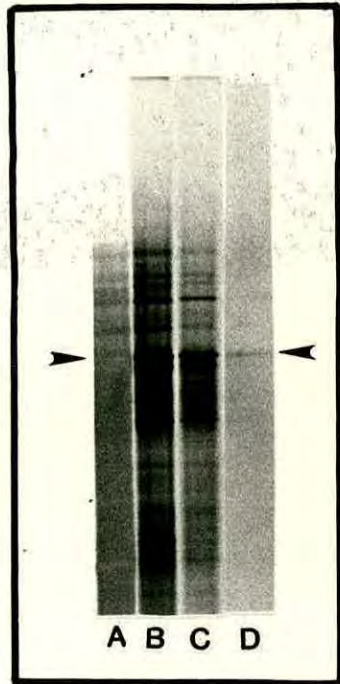


Figure 13 Purification of the 32,000 in vitro translation product using an affinity substrate specific for chitinase.

Total RNA extracted from ethylene-treated tissue was translated in vitro and the translation mixture run on an affinity column of regenerated chitin. The products which bound to the column were subsequently analysed by SDS-PAGE and fluorography. This is a similar procedure to that used to purify chitinase. The figure illustrates the different stages of the process. A, total translation products. B, sodium carbonate wash. C, sodium acetate wash. D, fraction bound to the column and eluted with acetic acid.

32 kD product was selectively bound by the regenerated chitin. As this is a very specific method, the result suggested a structural relationship between chitinase and the 32 kD translation product. Traces of the 42 kD band (cellulase) were obtained as contaminant. This would again seem to indicate a possible relationship between the 32 kD translation product and cellulase.

3.7.4 Comparison of the kinetics of induction of chitinase and the 32 kD product by ethylene

Chitinase has been shown to be induced in leaves by ethylene (Abeles et al . 1971; Boller et al . 1983) The continuous presence of ethylene was a necessary requirement for the full induction of the enzyme. Both observations are in agreement with the way the 32 kD translation product is induced during ethylene-accelerated abscission. Ethylene strongly induces the appearance of the 32 kD translation product within 6 h of the treatment (figures 5 and 15). When RNA was extracted from plants which had been incubated in ethylene and then transferred to hypobaric conditions, a treatment known to remove internal ethylene (Nilsen and Hodges, 1983), and translated in vitro the 32 kD product disappeared (figure 14). When ethylene was added back to the hypobaric chamber the 32 kD product reappeared. Thus, the continuous presence of ethylene was necessary for the induction of the mRNA coding for the 32 kD product.

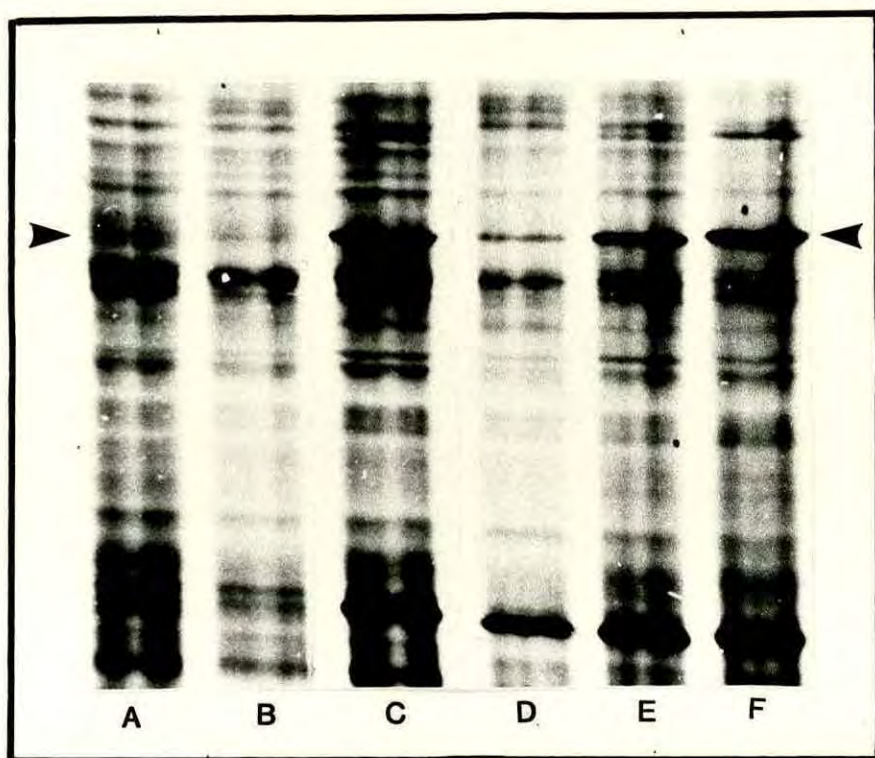


Figure 14 Effect of an hypobaric treatment on the synthesis and accumulation of chitinase mRNA in abscission zones as detected by in vitro translation.

Bean explants were excised and kept in hypobaric conditions for different periods of time in the absence or presence of ethylene. After the treatment, RNA was extracted and translated in the wheat germ lysate. The products were analysed by SDS-PAGE and fluorography. The lanes contain in vitro translation products from: A, Untreated tissue at normal pressure. B, Tissue kept in hypobaric conditions for 120 h without ethylene. C, Tissue kept for 18 h in ethylene at normal pressure. D, Same as C but with an additional incubation of 48 h in hypobaric conditions. E, Same as D but including 50 ppm of ethylene in the hypobaric chamber. F, Tissue incubated for 72 h in ethylene at normal pressure. 300,000 cpm were loaded per track. The arrows indicate the position of in vitro chitinase.

3.7.5 Partial proteolytic digestion

Attempts to compare by peptide mapping the structure of the 32 kD product and chitinase were unsuccessful. In the experiments with the 32 kD product, the results were ambiguous. Long exposures of the X-ray film were needed to visualize the digestion pattern, but the signal was so weak that no meaningful results could be obtained. Clearly, the major problem was to obtain enough radioactive 32 kD product. Consequently, no comparison could be made with the digestion profile of chitinase which was easily obtained. Modifications of the original method (Cleveland, 1983) did not produce satisfactory results.

3.7.6 Discussion

The aim of all these experiments was to ^{TEST}~~PROVE~~ the hypothesis that the 32 kD translation product, which appeared during the induction of abscission by ethylene in bean abscission zones, was chitinase. According to the results, there was a strong similarity between the two polypeptides. They seemed to share antigenic and structural similarity as indicated by the immunoprecipitation and affinity chromatography experiments. As chitinase is thought to be a secreted enzyme possessing a signal sequence (Boller and Vogelli, 1984), the 2 kD difference in mw between the two products would be explained by the presence of this sequence (Blobel and Dobberstein, 1975) which would be removed by microsomal membranes.

The kinetics of induction for the 32 kD product and chitinase are also comparable, as both products do not start accumulating before a lag of 6 h of ethylene treatment. The 32 kD translation product seems to be synthesized de novo as it cannot be detected at the beginning of the treatment. On the other hand, it might also be that the mRNA is present all the time but is not translatable until 6 h of ethylene treatment. The in vitro translation assay does not allow me to rule out either possibility. Chitinase induction has been shown to be inhibited by cycloheximide (Boller et al . 1983). This suggested that protein synthesis was required for the induction. There are suggestions, therefore, that the 32 kD product and chitinase are synthesized de novo on ethylene induction.

On the basis of all this evidence, it was tentatively concluded that the 32 kD translation product was chitinase. The determination of the amino acid sequence of both products and its comparison will confirm the identification; however, from now on for all practical purposes, the 32 kD in vitro translation product will be referred to as chitinase. Attempts to confirm this identity by peptide mapping were unsuccessful.

3.8 Regulation of chitinase levels by ethylene and auxin as detected by in vitro translation

To quantify the total amount of chitinase mRNA during ethylene induction, the bands seen in the figure 5 were scanned densitometrically. The intensity of each band

was used to calculate the relative percentage of chitinase in relation to the rest of the translation products . These are plotted as a function of time of ethylene incubation (figure 15). The relative amounts of chitinase increase steadily within 6 h and reach a maximum by 12 h. Chitinase made up about 8% of the total translation products on ethylene induction. In the absence of ethylene, the proportion of chitinase remained at basal levels. The levels of the 32 kD product between 0 and 6 h of ethylene treatment were not examined. Therefore the extrapolation made in the figure 15 for the levels of the 32 kD product during the first 6 h of ethylene induction should be viewed with caution. I am assuming that the increase in the 32 kD product levels is uniform for that period of time, but this may not be the case.

The presence of ethylene seemed to be essential throughout the process since the removal of ethylene by incubation in hypobaric conditions caused chitinase mRNA levels to drop (figure 14). If ethylene was added back to the hypobaric chamber, chitinase synthesis would start again.

As opposed to the effects of ethylene on chitinase, auxin had no effect on chitinase mRNA, as judged by the in vitro translation assay. When auxin was applied to abscission zones, no effect on the chitinase induction could be observed (figure 16). This lack of effect of auxin on chitinase was apparent regardless of the length

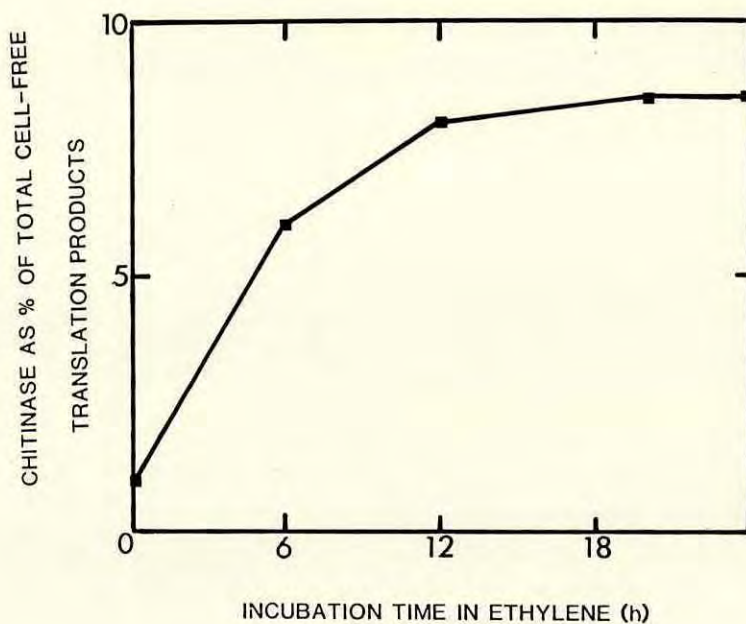


Figure 15 Changes in the levels of translatable chitinase mRNA in proportion to the total translation products from ethylene treated-abscission zones

Chitinase synthesis relative to total protein synthesis in vitro was calculated by a densitometry scan of the fluorograph shown in the figure 5. The percentage of chitinase message was calculated for each point in the time course and expressed as a percentage in relation to total translation products.

of time of application of auxin or the presence of ethylene (figure 16). Chitinase was still induced by ethylene in the presence of auxin.

It is known that auxin is a potent inhibitor of abscission in beans (Horton and Osborne, 1967; Addicott, 1970; Sexton et al . 1980). The fact that auxin did not have an effect on chitinase, suggests that chitinase does not play a specific or direct role in the process of abscission. And if it does, it is not related to the effects of auxin on the plant.

Auxin did have an effect on the 42 kD product, possibly cellulase (figure 16), which is thought to be the main enzyme involved in abscission in beans (Horton and Osborne, 1967; Sexton and Roberts, 1982).

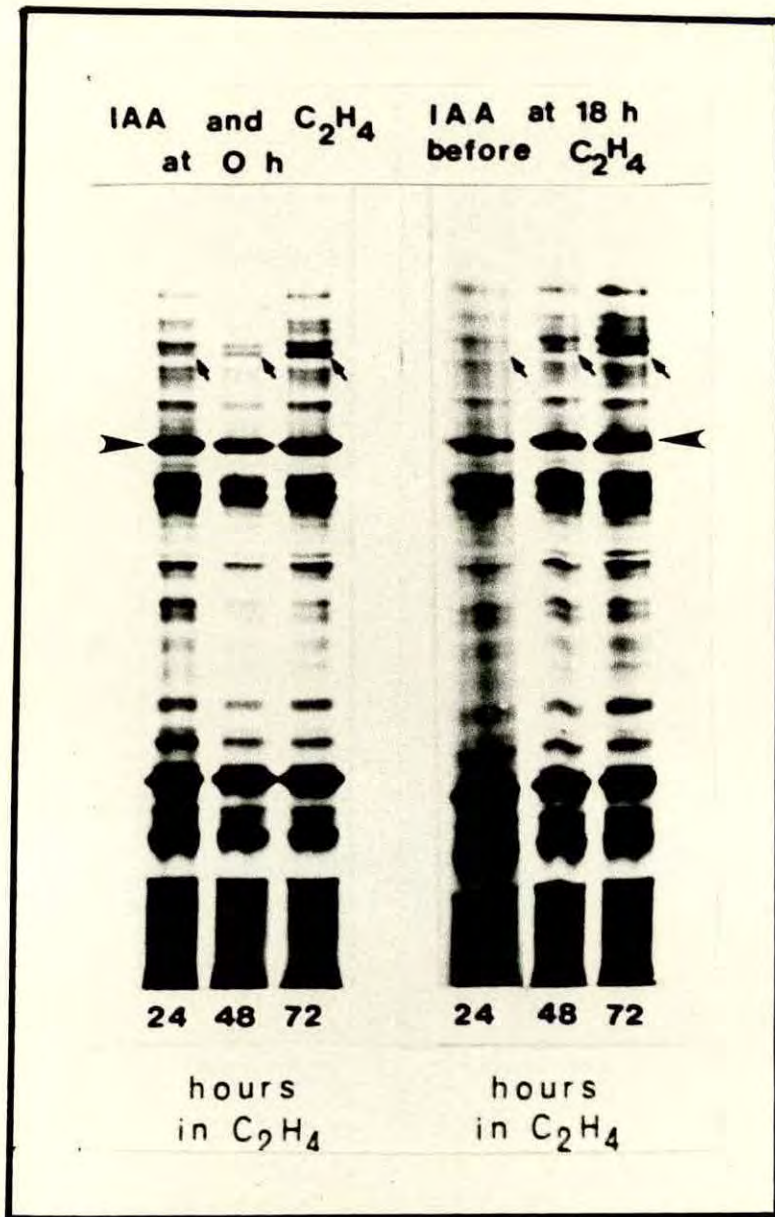


Figure 16 Effect of auxin application on the synthesis and accumulation of chitinase mRNA in abscission zones.

Bean explants were treated with ethylene for different periods of time in the absence or presence of 0.0001 M IAA, which was applied to the explants. Auxin was applied simultaneously or before the incubation in ethylene. The abscission zones were excised and RNA extracted and translated in the wheat germ lysate. The translation products were analysed by SDS-PAGE and fluorography. About 400,000 cpm were loaded per track. The arrows indicate the position of cellulase (top) and chitinase (bottom).

CHAPTER 4 INDUCTION OF CHITINASE BY ETHYLENE AS MEASURED BY ENZYMATIC ACTIVITY

4.1 Rationale

As part of the attempt to ^{test}~~prove~~ the hypothesis that the 32 kD translation product was chitinase, I set out to purify chitinase from abscission zones using a protocol identical to that of Boller et al . (1983). This was followed by the demonstration that chitinase was present in an active form. These were essential requirements to prove or disprove the above hypothesis. This section describes the purification of chitinase from abscission zones and the development of an assay for chitinase. It also describes studies on the induction of chitinase by ethylene in different organs of the bean plant as assayed by enzymatic activity and immunological experiments.

4.2 Purification of chitinase from bean abscission zones

Following a protocol identical to that of Boller et al . (1983) chitinase was purified from abscission zones. The figure 17 shows the progress of purification. The enzyme was essentially homogeneous as judged by SDS-PAGE and had an apparent molecular weight of 30 kD., which is the same as chitinase from bean leaf. The development of an assay (see next section) allowed the purification of chitinase on the basis of activity. The table 2 shows the 17-fold purification of chitinase on the basis of its specific activity.

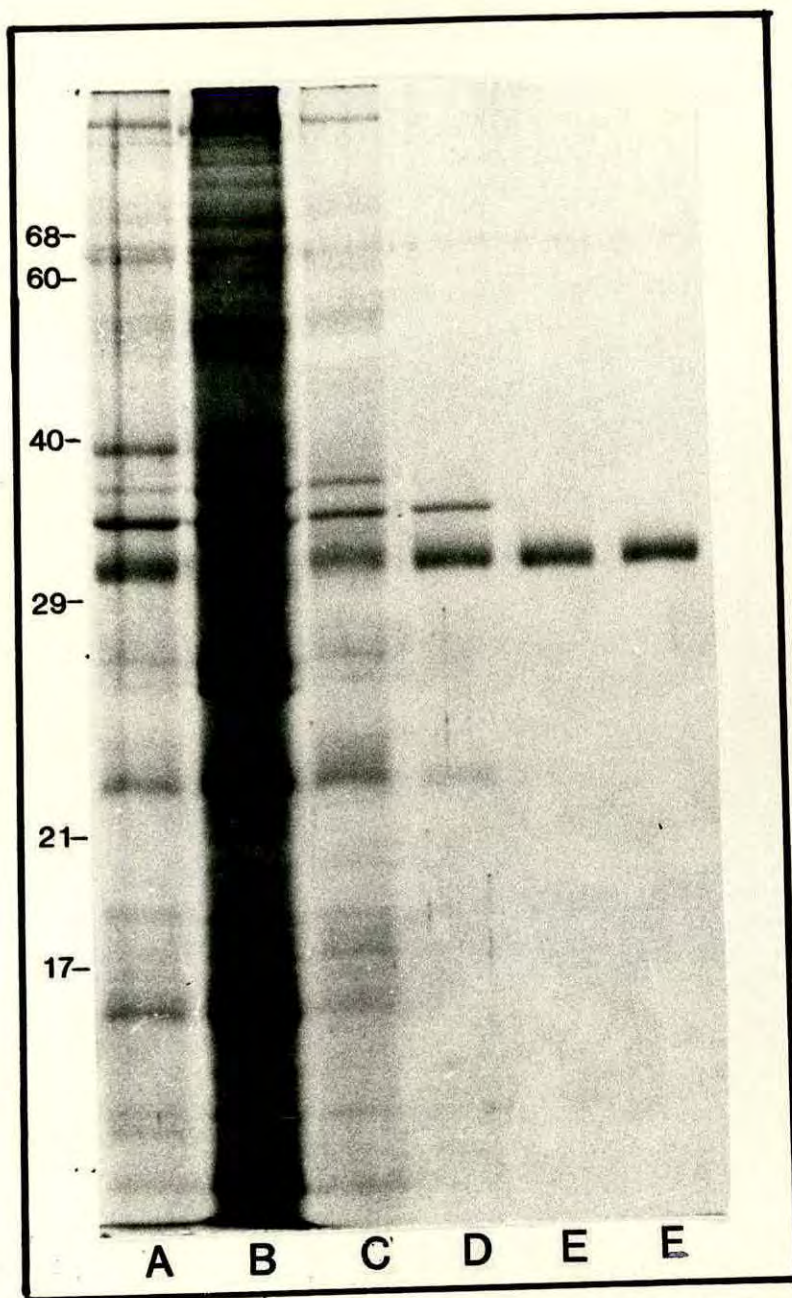


Figure 17 Progress of purification and homogeneity of chitinase on SDS-PAGE

Chitinase was purified from abscission zones according to Boller et al . (1983). Fractions from each stage of purification were subjected to SDS-PAGE on 12% slab gels. About 40 ug of protein were loaded in tracks A and C. Track B was overloaded (80 ug) to show the specificity of the affinity column (regenerated chitin) to bind chitinase. About 10 ug of protein were loaded in tracks D and E. The tracks contain: A, crude homogenate from abscised tissue. B, fraction that did not bind to the affinity column. C, sodium carbonate wash. D, sodium acetate wash. E, fraction bound to the column and eluted with acetic acid. The numbers on the left correspond to molecular weight markers in kilodaltons.

Table 2 Purification of chitinase from bean abscission zones

| Fraction | Total Protein (mg) | Total Chitinase (mU) | Specific activity (mU/mg) | Yield (%) |
|-----------------------------------|-----------------------|-------------------------|------------------------------|-----------|
| Crude extract | 178 | 170,610 | 958 | 100 |
| Supernatant after 50°C incubation | 98.5 | 161,476 | 1639 | 95 |
| Precipitate in ammonium sulphate | 62 | 156,948 | 2531 | 92 |
| Chitin column eluate | 1 | 16341 | 16341 | 10 |

Chitinase was found not only in leaves as reported by Boller et al . (1983) and abscission zones but in every organ tested. The enzyme seemed to be identical as indicated by SDS-PAGE and immunological evidence (see below). If chitinase has a defensive role in plants, then it was not surprising to find it all over the plant. No attempts were made to test bean roots for chitinase activity.

4.3 Development of an assay for chitinase

Chitinase is relatively difficult to assay since its natural substrate (chitin) is insoluble in water. Ordinary methods to assay chitinase are based on the estimation of substrate degradation, like the viscometric (Tracey, 1955; Otakara, 1961) or turbidimetric (Jeuniaux, 1966) methods, or the measurement of end products, like the colorimetric (Reissig et al . 1955) or radiometric (Molano et al . 1979) methods.

In order to find a suitable assay in terms of reproducibility, sensitivity and accuracy, two different assays were tested, one viscometric (Tracey, 1955; Otakara, 1961) and the other radiometric (Molano et al . 1977).

4.3.1 Viscometric assay

This assay consists in the measurement of the reduction in viscosity of a soluble substrate by chitinase. Chitosan acetate (Tracey, 1955) and glycol chitin (Otakara, 1961) were tested as soluble substrates.

Chitosan was found to be the best substrate. The results were reproducible and comparable to those of the radiometric assay (see below). Using this assay, it was possible to measure the induction of chitinase activity by ethylene. However, even though the assay was reproducible enough using a particular batch of substrate, it was found that absolute measurements varied from batch to batch of substrate. This was also the case with glycol chitin. The assay was performed up to three times on the same sample but the results were not comparable if two different batches of substrate were used. For this reason I did not find the assay satisfactory and in addition it was time consuming taking 1 h to assay each sample.

It is known that with viscometric assays the absolute rate of decrease in viscosity may vary with the mode of preparation of the substrate and that these assays are not particularly reproducible (Jeauniaux, 1966). For that reason they are not recommended for standardization of chitinase measurements (Jeauniaux, 1966).

4.3.2 Radiometric assay

This assay is based on the fact that chitin is not soluble in water whereas the degradation product released by chitinase (diacetyl chitobiose) is soluble. The chitinase present in bean has been shown to be an endochitinase (Boller et al . 1983). The ultimate end-product released by chitinase is chitobiose. Endochitinases cannot hydrolyse chitobiose (Jeauniaux,

1966).

The preparation of the radioactive substrate (regenerated chitin) involved acetylation of chitosan with tritiated acetic anhydride (Molano et al . 1977). The tritiated chitin was a generous gift of Tom Boller. The soluble products released by chitinase were radioactive and could therefore be easily detected by liquid scintillation counting. The assay was found to be very reproducible. The radiometric assay has been reported to have a sensitivity comparable to that of the colorimetric assay (Mauch et al . 1984) which is the most commonly used assay for chitinase (Abeles et al . 1971; Boller et al . 1983; Wadsworth and Zikakis, 1984). The radiometric assay was therefore chosen as the standard assay for its sensitivity, reproducibility and rapidity. Up to 60 samples could be analyzed in 2 h.

4.4 Estimation of chitinase activity

A plot of the soluble product formed as a function of added enzyme did not yield a straight line (figure 18a). The lack of linearity of the reaction of chitinase with its substrate has been interpreted (Molano et al . 1977) as a possible time-dependent change in the substrate. Addition of fresh enzyme to the reaction mixture increased the rate only slightly, suggesting that a modification had taken place in the substrate (Molano et al . 1977).

A dilution series of the enzyme was prepared (as suggested by Boller et al . 1983), therefore, and the

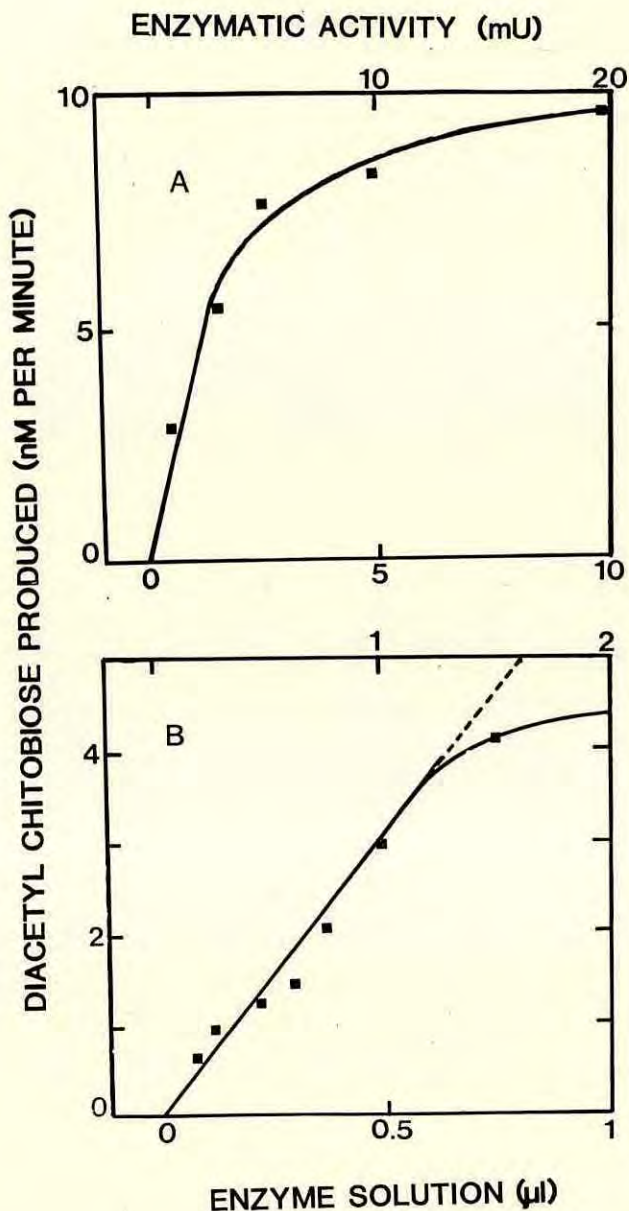


Figure 18 Product formation as a function of enzyme concentration

The radiometric assay was used for the construction of these curves. The reaction mixture consisted of enzyme, 20 mM phosphate buffer pH 6.3 and 1.5 mg of tritiated chitin (162 nCi/mg) in a final volume of 0.25 ml. The reaction was stopped after 30 min with 0.25 ml of 10% TCA. After centrifugation (12,000 \times g for 10 min), the radioactivity of 0.25 ml of the supernatant was determined. The scintillant used was 0.7% butyl-PBD in 1:1 triton X 100-toluene (v/v). As the reaction was not linear (A) a dilution series of the enzyme was prepared and a curve constructed (B). The initial slope of B (broken line) was used for calculation of enzyme units

activity determined for enzyme concentration approaching zero (figure 18b). The initial slope was used for calculation of enzyme units.

4.5 Induction of chitinase by ethylene

4.5.1 Time course of enzymatic activity

Chitinase was extracted from abscission zones and assayed radiometrically. During ethylene treatment, chitinase activity could be detected after a lag of about 6 h. This activity increased reaching by 30 h a value about 30-fold higher than that of untreated plants (figure 19). Some chitinase activity could be detected within the first 6 h of induction. This low level of activity was maintained in the untreated tissue but there was no increase in activity with time. These results were in complete agreement with those of Boller et al . (1983) who also showed that there was a lag of 6 h before chitinase started accumulating in bean leaves.

A similar time course of induction was also exhibited by chitinase protein as assayed by western blotting (see below). In this case, chitinase started to accumulate after a lag of 6 h, the increase being constant right through 36 h. In the absence of data on the accumulation of chitinase mRNA within the first 6 h of ethylene induction it is difficult to establish whether accumulation of chitinase mRNA is a prerequisite for the expression of chitinase activity. The synthesis and expression of mature chitinase may be close events in

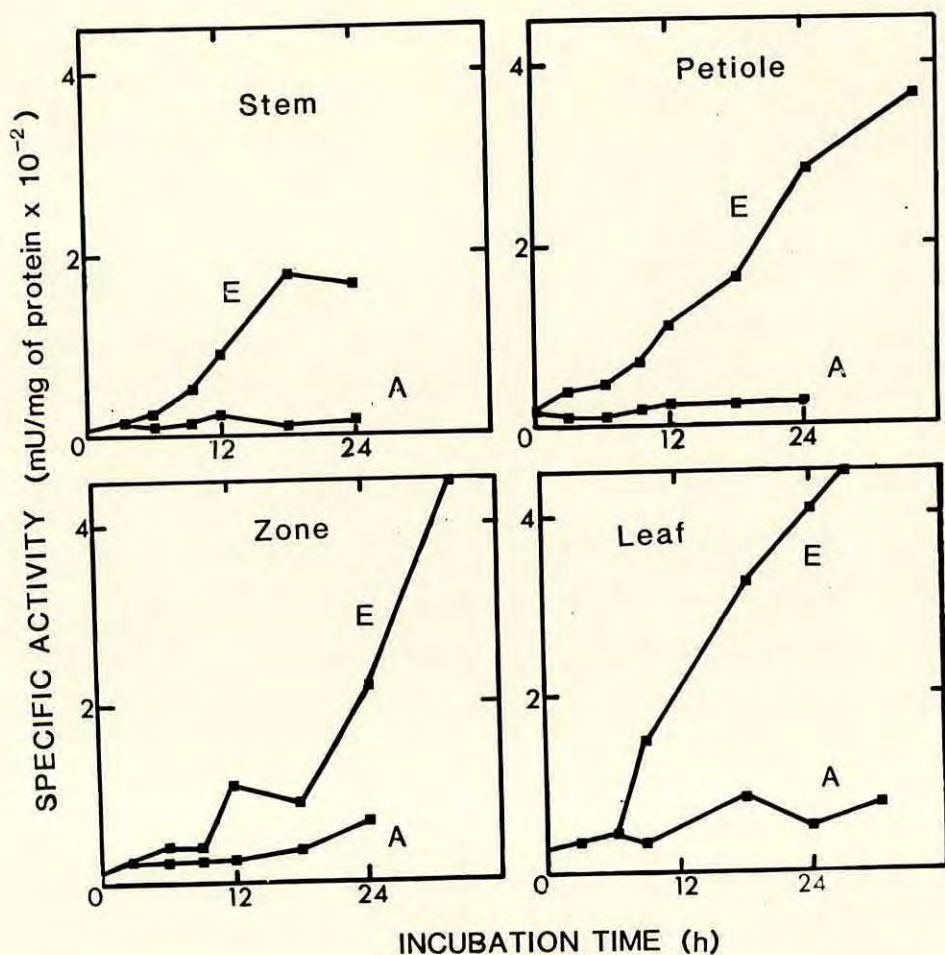


Figure 19 Kinetics of chitinase accumulation in different organs as detected by enzymatic assays

Bean seedlings were incubated in air or ethylene for different periods of time. The organs were excised from the explants and a crude homogenate prepared from each. The homogenates were then assayed for chitinase activity. Each point in the graphs represents the mean of at least three determinations. E= time course in ethylene. A= time course in air.

time.

The time course of chitinase induction by ethylene in abscission zones was identical to that of petioles and stems, except that in the case of stems the increase reached a lower value by 36 h (figure 19). In leaves, on the other hand the induction followed a slightly different pattern (figure 19). The increase in chitinase activity was more pronounced after 6 h and reached higher values than in any other organ. The untreated leaves did not show a comparable accumulation of chitinase. The results obtained with leaves are comparable to those obtained by Boller et al . (1983).

The dose of ethylene used in this study (50 ppm) was five times higher than that saturating since a half maximal induction of chitinase can be achieved with 0.1 ppm and a saturating concentration is 10 ppm (Abeles and Ganahan, 1968; Boller et al . 1983). Even though chitinase can be induced by ethylene as shown by others (Abeles et al . 1970; Boller et al . 1983; Mauch et al . 1984) and in this study, the presence of ethylene is not a necessary requirement for induction. It has been shown that chitinase can also be induced by chitosan, actinomycin D, cadmium ions, fungal elicitors and concentrated salt solutions (Mauch et al . 1984; Metraux and Boller, 1985). In cucumber leaves, chitinase increased 600-fold in response to infection by fungal elicitors (Metraux and Boller, 1985).

When chitinase is induced by ethylene, the continuous presence of the latter is essential for induction since the removal of ethylene causes a halt in the synthesis (Boller et al . 1983 and figure 14)

4.5.2 Western blotting

To determine if the appearance of chitinase protein correlated with the increase in enzymatic activity, protein extracts were prepared from tissue treated for different periods of time. The crude extracts were separated on SDS-PAGE, blotted on to nylon membranes and then challenged with chitinase antiserum. The bound antibodies were detected by radiolabelled protein A. The result of this experiment is shown in the figure 20. The antiserum only recognized one band on the filters. The appearance of chitinase activity correlates closely with the appearance of chitinase antigen and, presumably, synthesis of the polypeptide as judged by this method. In every organ tested (abscission zone, leaves, petioles etc) chitinase antigen was first detectable by 6-9 h after ethylene induction and it accumulated thereafter. Although some chitinase activity can be detected within 6 h of ethylene treatment, no chitinase antigen was detectable within that range even after long exposures of the filters. There are, at least, two explanations for this result; enzymatic assays are more sensitive than immunoblotting or the enzymatic activity detected within the first 6 h of ethylene treatment is due to another chitinase enzyme



Figure 20 Chitinase accumulation in leaves and zones as detected by western blots

Bean explants were incubated with ethylene for different periods of time. Abscission zones and leaves were then excised and a crude homogenate prepared from each sample. An aliquot of the homogenate was run on SDS-PAGE and subsequently transferred on to nitrocellulose. The nitrocellulose was incubated with chitinase antibodies, washed and incubated with radioiodinated protein A. After washing, the filters were exposed to pre-flashed X-ray film at -70°C . The lanes contain products precipitated by chitinase antiserum from tissue treated with ethylene for A, 0 h. B, 2 h. C, 6 h. D, 9 h. E, 12 h. F, 24 h.

which does not cross-react antigenically with the 30 kD protein.

CHAPTER 5 ATTEMPTS TO OBTAIN A CLONE TO BEAN CHITINASE: PREPARATION OF A cDNA LIBRARY TO mRNA FROM ABSCISSION ZONES.

5.1 Rationale

In order to study many of the processes involved in the regulation of gene expression by ethylene and other growth substances, the availability of defined gene probes is an essential prerequisite. Such probes will allow an investigation of the expression of many ethylene-regulated genes, the level of regulation by ethylene, the elucidation of upstream sequences regulated by ethylene and the organization of these genes in the nucleus.

The isolation of cDNA clones to mRNA molecules is usually the first step towards the isolation of the genes themselves. The construction of the library was, therefore, undertaken in the hope of identifying a cDNA clone containing a chitinase sequence.

5.2 Aims

The aims for constructing the library was to identify and characterize a specific cDNA clone to chitinase which could then be used

- a) As hybridization probe to assess chitinase mRNA levels during ethylene induction.
- b) To screen a lambda genomic library of bean and identify the corresponding nuclear gene.
- c) To study the organization of the chitinase gene in the nucleus and characterize it.

5.3 Construction of the cDNA library

The library was constructed by D. Baulcombe (P.B.I., Cambridge) and P. Kelly (BioScot, Edinburgh) according to the method of Wickens et al . (1978) as modified by Baulcombe and Key (1980). Poly A+ RNA was used as template. The first strand of the cDNA was primed by oligo dT hybridized to the 3' poly A+ tail of the mRNA. Avian Myeloblastosis Virus (AMV) reverse transcriptase was used to copy the mRNA sequences, extending the oligo dT primer. Following hydrolysis of the mRNA, single stranded cDNA has the ability to form a hairpin loop at the 3' end and so prime the synthesis of a complementary second strand by E. coli DNA polymerase I (Efstratiadis and Villa-Komaroff, 1979). The hairpin loop was removed by S1 nuclease digestion and the double stranded cDNA was sized fractionated by sucrose gradient centrifugation. Poly (dC) tails were added to the size selected double stranded cDNA by using terminal deoxynucleotide transferase. Poly (dG) tails were added to Pst 1-cut pBR 322 and vector and cDNA annealed. This regenerated the Pst 1 site in the hybrid plasmid. The plasmid was used to transform E. coli LE 392 (Maniatis et al . 1982). As the cDNA was inserted into the Pst 1 site of pBR 322, The ampicillin resistant gene was inactivated. Colonies containing recombinant DNA plasmids were identified by the phenotype tetracycline-resistant, ampicillin-sensitive.

5.4 Characteristics of the library

About 1000 clones containing recombinant plasmid were obtained. The annealed cDNA/pBR 322 transformed into E. coli LE 392 gave rise to colonies at a frequency of 4.7×10^5 per ug of DNA. Tailed vector alone gave rise to about 350 colonies per ug of DNA and no colonies were observed in the absence of exogenous DNA.

5.5 Strategies for screening cDNA libraries

A number of approaches have been developed which exploit particular properties of the sequences to be identified. Some of these are outlined below:

5.5.1 Colony hybridization (Grunstein and Hogness, 1975)

Bacterial colonies harbouring recombinant plasmids are grown on nitrocellulose filters. The colonies are lysed in situ and the DNA immobilised on the filters which are then hybridized to a probe. The probe employed will depend on the nature of the gene of interest. Organ-specific or developmentally-regulated genes can be identified by a +/- hybridization. Labelled RNA from induced (+) and non-induced (-) cells can be used to probe duplicate filters and the clones giving a strong signal with a particular RNA selected for further analysis. If a partial amino acid sequence of the gene product is known, synthetic oligonucleotides corresponding to the predicted sequence may be employed. Alternatively, a clone of the homologous gene from another organism can be used to probe the library. This

method is particularly suitable as a rapid screening to detect fairly abundant sequences and it is more specific than the other methods. However, a sequence present in low abundance or underrepresented in the population may be undetected by this method.

Grunstein and Hogness (1975) point out that in their method the extent of hybridization is not greatly altered by a 1000-fold reduction in the concentration of the RNA probe. This might suggest that differences in mRNA abundances between 'depleted' and 'enriched' probes of less than a 1000-fold may not be sufficient to be detectable when comparative colony hybridizations are attempted. Also, unspecific hybridizations to bacterial debris on the filters may produce false positives.

5.5.2 Mini lysates (Barnes, 1977)

This method in essence is similar in nature to the previous one. Colonies are grown on LB plates and mini lysates are made from each. The lysates are fractionated on an agarose gel and the DNA transferred on to nitrocellulose (Southern, 1975). The filters are then probed using one of the probes mentioned previously. This method is more specific than colony hybridization as it permits a more direct analysis of each clone, allowing a close examination of the inserts. Sequences underrepresented might also pass undetected.

5.5.3 Hibrid-release translation (Ricciardi et al . 1979)

DNA from individual recombinant clones transferred to

nitrocellulose filters can be used to 'hybrid select' a complementary mRNA which is subsequently eluted and translated in a cell-free system. If an antibody is available, the translation products can be immunoprecipitated. This method is often used to confirm the identity of clones selected after previous rounds of screening like the ones mentioned above.

5.5.4 Immunological screening (Broome and Gilbert, 1978)

If the cDNA is cloned into an expression vector in the correct orientation and reading frame, an antibody can then be used as a probe to detect the product which is usually expressed as a fusion protein within the bacterium

5.6 Rationale for screening the cDNA library by differential hybridization

The fact that some genes exhibit developmental or organ specific expression or are inducible by environmental factors, makes possible identification of their cDNA clones by differential hybridization. The method consists of screening the library in duplicate with end-labelled poly A⁺ RNA from induced and non-induced cells or from different organs. The colonies which hybridize to the desired type of poly A⁺ RNA are selected for further analysis.

This approach has been taken before to identify clones containing cDNA's to mRNA's induced during development (Williams and Lloyd, 1979), by light (Thompson et al .

1983) or by PGS (Baulcombe and Buffard, 1983; Christoffersen et al . 1984). For all these reasons, this was the approach taken in this study. Since the colony hybridization method of Grunstein and Hogness (1975) has several inconveniences (see above) the miny lysate method of Barnes (1977) was employed for the primary screening. This method is reckoned to be more unequivocal (Baulcombe and Buffard, 1983; Forde, 1983) and in addition gives an indication of the approximate size of the cDNA insert.

5.7 Primary screening by differential hybridization

Most of the work in this chapter was carried out together with P.Kelly. Clones containing recombinant plasmids were grown on LB plates. The clones showing the phenotype tetracycline-resistant, ampicillin-sensitive were selected and crude nucleic acid extracts made from each according to Barnes (1977). After electrophoresis, blotting on to nitrocellulose and denaturation of the DNA, the filters were hybridized to end-labelled poly A+ RNA from ethylene-treated tissue only. This procedure selected clones containing cDNA's to abundant RNA's. In the first round of screening, 76 clones (out of 1000) giving a strong hybridization signal were picked (figure 21). These clones were processed in an identical manner as described and hybridized, in duplicate, to poly A+ RNA from both control and ethylene-treated tissue. 30 clones hybridized preferentially to RNA from ethylene-treated tissue. These clones were selected and

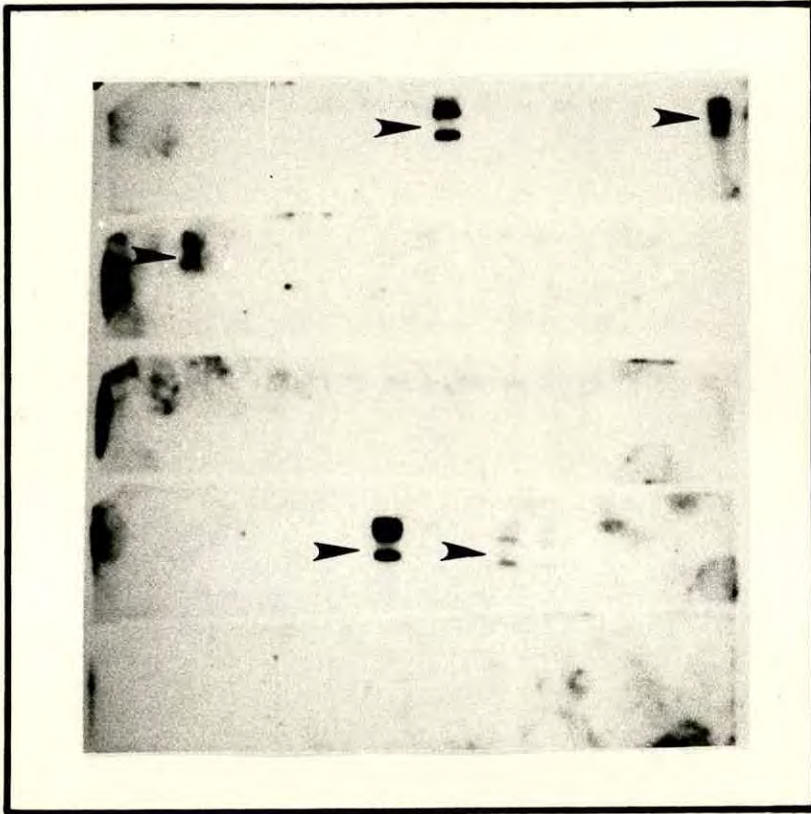


Figure 21 Screening of a cDNA library by Southern blot analysis of colony lysates and hybridization to RNA from ethylene-treated tissue.

Colony lysates of cDNA clones were prepared according to the method of Barnes (1977). Each lysate was then separated on a 1.2% agarose gel and the plasmid-DNA region of the gel was transferred on to nitrocellulose. The clones were subsequently hybridized to end-labelled RNA extracted from ethylene-treated abscission zones. The filters were washed and autoradiographed at -70°C . The figure illustrates some nitrocellulose filters containing hybridizing clones (arrows).

subsequently digested with Pst 1 to excise the insert and the products analysed by agarose gel electrophoresis. Clones containing inserts above 400 base pairs were selected (18 clones).

5.8 Hybrid release translation

This is a very sensitive method which can be used to detect mRNA's of even low abundance (0.1% of total RNA) (Ricciardi et al . 1979). The SDS-PAGE gel analysis of in vitro translation products of abscission zones indicated that chitinase mRNA was one of the most abundant sequences from tissue incubated in ethylene (see figure 5). It was then reasonable to expect that at least one of the 18 clones selected would contain a chitinase sequence. In order to confirm this hypothesis the hybrid release translation method was undertaken.

Plasmid DNA from the 18 clones was immobilised on a nitrocellulose filter and hybridized to poly A+ RNA from ethylene induced tissue. The filter was washed-free of non-hybridized RNA and the specifically hybridized RNA was eluted (released) and ethanol precipitated. The RNA was then translated in the wheat germ system and the products analysed by SDS-PAGE.

Of the 18 clones selected by previous methods only one (pABS 17) gave a single polypeptide of mw 32,000 on in vitro translation (figure 22). This result was consistently obtained.

Was this a clone to chitinase? An antibody to chitinase was not available at that time to confirm this result by

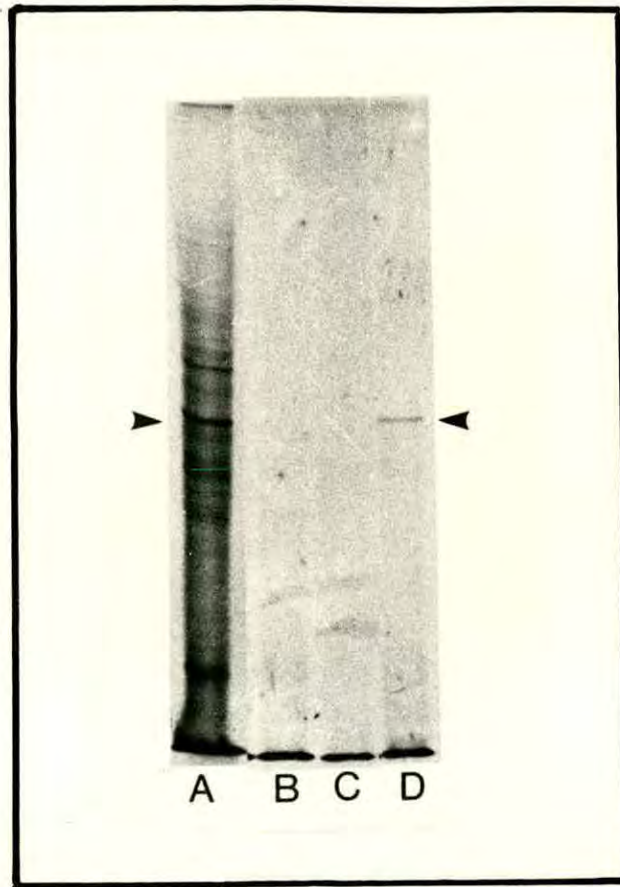


Figure 22 In vitro translation of hybrid-selected mRNA by pABS 17

Total RNA was extracted from ethylene-treated abscission zones and hybridized to nitrocellulose-bound plasmid DNA from pABS 17. The filters were washed and the bound RNA was released and translated in the wheat germ lysate. The products were analysed by SDS-PAGE and fluorography. The lanes in the figure correspond to A, total translation products. B, products selected by the vector pBR 322. C, products selected by the nitrocellulase filter without DNA. D, products selected by pABS 17. The arrows indicate the position of the 32,000 in vitro translation product.

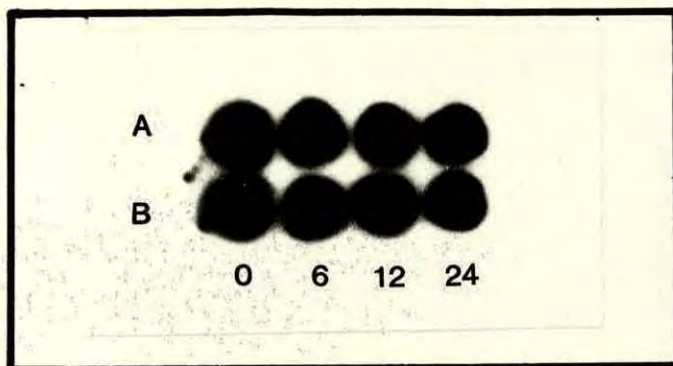


Figure 23 RNA dot blots hybridized with labelled DNA from pABS 17

Bean explants were incubated in air or ethylene for different periods of time. Abscission zones were excised and RNA extracted and spotted (10 ug in each case) on to nitrocellulose filters. The RNA was then hybridized to nick-translated DNA from pABS 17. The filters were then washed and exposed to pre-flashed X-ray film at -70°C . The letters correspond to: A, time course in ethylene. B, time course in air. The numbers refer to the time of incubation in hours.

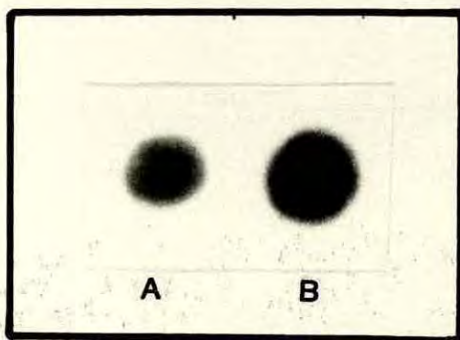


Figure 24 DNA dot blots hybridized with labelled DNA from pAT 71

5 ug of plasmid DNA from pABS 17 and pAT 71 were spotted on to nitrocellulose filters. The DNA was then hybridized to the nick-translated insert of pAT 71. The filters were washed and exposed to preflashed X-ray film. A, pABS 17. B, pAT 71.

hybrid release translation and immunoprecipitation. So we decided, as a first approach to answering this question to use pABS 17 in RNA blot experiments, using RNA from control and abscised tissue. We knew from in vitro translation results that chitinase mRNA was not detectable until 6 h after ethylene induction. If this was a transcriptional regulation, we would not expect to detect the mRNA in control tissue. However ethylene may also have post-transcriptional and translational effects (Jacobsen and Higgins, 1978).

Plasmid DNA was prepared from pABS 17 (Birnboim, 1983), digested with Pst 1 and the insert nick translated (Maniatis, et al . 1982). Total RNA was extracted from ethylene-treated and control tissue, spotted on to nitrocellulose and baked. The labelled insert was then hybridized to the RNA on the filters. The results of this experiment was ambiguous (figure 23). A very strong signal was obtained with RNA from both control and ethylene-treated tissue. This result was reproducible and suggested, if pABS 17 contained chitinase sequences, a post-transcriptional regulation of chitinase by ethylene. The mRNA would be present before the induction but not translated.

As hybridization to ribosomal RNA (rRNA) sequences, which are present constitutively, was a possibility I decided to hybridize pABS 17 to pTA 71 (a kind gift from S. Smith), a clone containing a full-length DNA ribosomal repeat unit from wheat (Gerlach and Bedbrook,

1979). I spotted some pABS 17 on nitrocellulose, baked it and hybridized it to the nick translated insert of pAT 71. There was a strong and reproducible hybridization signal (figure 24). This suggested homology between pABS 17 and ribosomal sequences and would explain the result obtained in the RNA blot experiments. This result had to be reconciled with that obtained in the hybrid release experiment. A mixed clone, containing sequences of chitinase and ribosomal RNA, was a possible explanation. I, then, digested the insert of pABS 17 with three different restrictions enzymes and following electrophoresis and transfer to nitrocellulose, hybridized it to the labelled insert of pAT 71. As figure 25 shows, pAT 71 hybridized to fragments of pABS 17 differentially. Some fragments showed a strong hybridization signal whereas others did not. This result suggested that either pABS 17 had sequences of both chitinase (as indicated by the hybrid release experiment) and rRNA (as indicated by the hybridization to pAT 71) or that parts of the chitinase coding sequence cloned in pABS 17 shared strong sequence homology with rRNA. In either case the resultant cDNA was not suitable for my studies. The strong hybridization signal of pABS 17 to ribosomal sequences would obscure any genuine hybridization in experiments to quantitate the changes in chitinase mRNA level that occur during ethylene induction. As none of the remaining 17 positive clones seemed to contain an insert

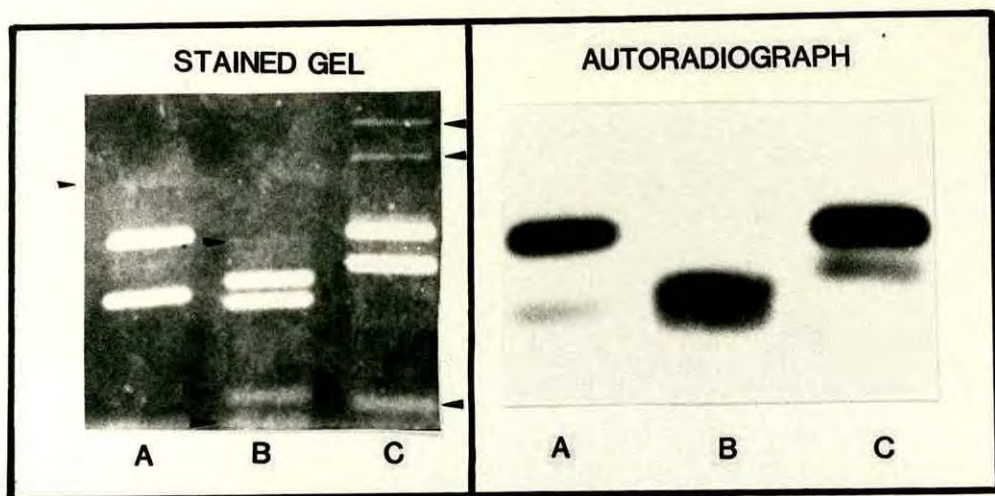


Figure 25 Hybridization of the insert of pABS 17 digested with three restriction enzymes to nick-translated pAT 71

Plasmid DNA was purified from the pABS 17 cDNA clone. The insert was excised and digested separately with three restriction enzymes. The products were run on a 1% agarose gel and blotted on to nitrocellulose. The filter was hybridized to nick-translated pAT 71, washed and exposed to pre-flashed X-ray film. The lanes correspond to the digests with the enzymes: A, Alu I. B, Hae III. C, Taq I. The arrows indicate the restriction fragments which did not hybridize to pAT 71.

with chitinase sequences, it was decided to undertake the construction of another cDNA library.

5.9 Construction of a second cDNA library

This second library was prepared by P. Kelly (BioScot) using poly A⁺ RNA from ethylene-treated tissue as template. The cDNA was synthesised essentially according to Wickens et al (1978) following an identical procedure as described in previous sections. After annealing with pBR 322 and transformation of E. coli LE 392, about 2000 clones containing recombinant plasmids were identified by the phenotype tetracycline-resistant and ampicillin-sensitive.

5.10 Screening of the second cDNA library

The clones were transferred to nitrocellulose and differentially hybridized to RNA from ethylene-treated tissue and untreated tissue as before. 93 clones were selected as hybridizing to RNA from ethylene treated tissue. The clones were size selected and those containing inserts larger than 600 base pairs (16 clones) were picked out. Hybrid release experiments were subsequently performed on each clone. The clones were transferred to nitrocellulose, hybridized to RNA from ethylene-treated tissue and the RNA that bound to the filter was released and used for in vitro translation. As an antibody was available this time, an immunoprecipitation was performed immediately after in vitro translation. The products were analysed by SDS-PAGE and fluorography. 12 out of the 16 clones

appeared to select a translation product of 32kD which was immunoprecipitated by the chitinase antibody (figure 26). Was any of this clones to chitinase? A repetition of this experiment without the immunoprecipitation step did not produce any clear-cut result (figure 27). No bands of 32kD mw could be detected. The 12 positive clones were then cross-hybridized with each other. The clones were digested with Pst 1 and fractionated by agarose gel electrophoresis. The DNA of the 12 clones was transferred on to nitrocellulose and hybridized in group to the insert of one of them. Using this method the clones were classified into two groups. A representative clone of each group (pAK 3 and pAK 11), containing the biggest insert, was chosen and employed in RNA blot experiments.

RNA from control and ethylene-treated tissue was fractionated on a denaturing agarose gel and subsequently blotted on to nitrocellulose (Thomas, 1980). Filters were then hybridized in duplicate to pAK 3 and pAK 11. There was a strong hybridization signal with RNA from induced and non-induced tissue when pAK 11 was used (figure 28). With pAK 3 the results were more ambiguous. The clone seemed to hybridize to sequences present in ethylene-treated and control tissue but the signal in control tissue was weaker than that of induced tissue (figure 29). These results were reproducible. In dot-blot experiments, both clones also seemed to hybridize although with different intensity to sequences

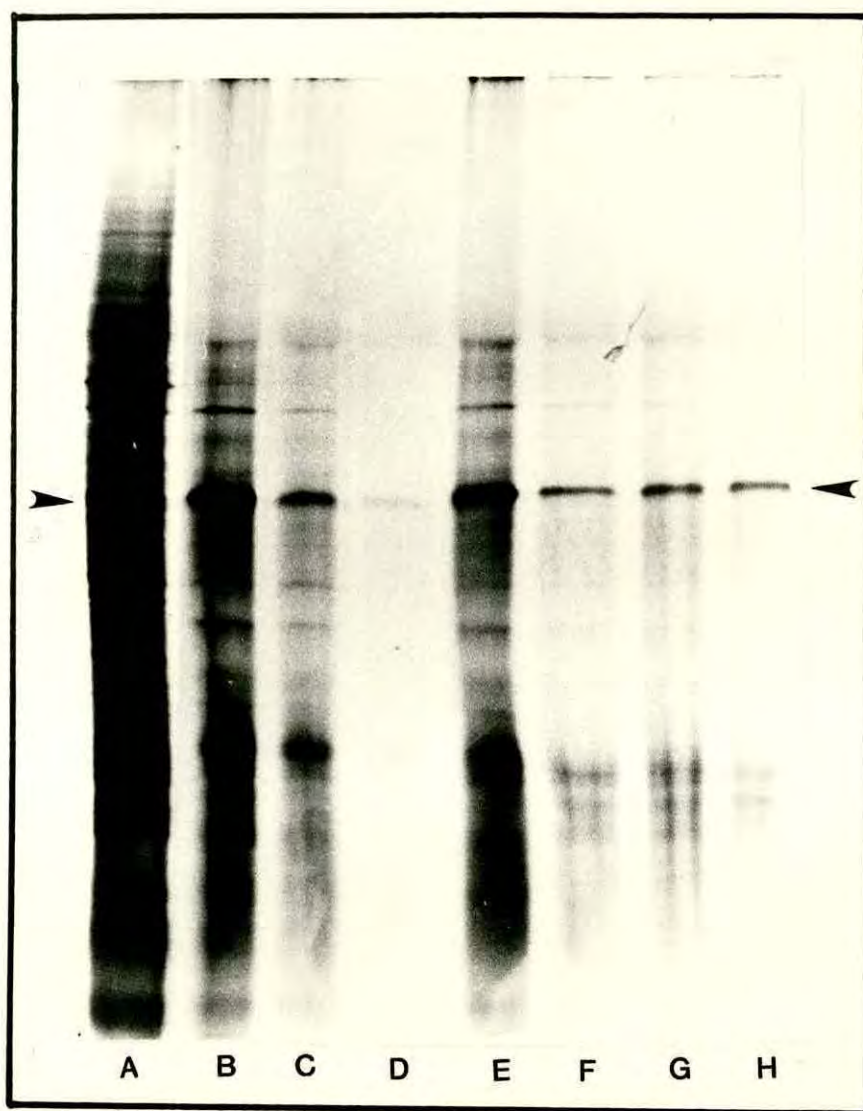


Figure 26 In vitro translation of hybrid-selected mRNA's and immunoprecipitation using chitinase antiserum.

RNA from ethylene-treated tissue was hybridized to nitrocellulose-bound DNA from different cDNA clones. Bound RNA was released and translated in the wheat germ lysate. After translation, chitinase antiserum was added and the mixture incubated overnight at 4°C. Protein A-Sepharose was added and after 1 h at room temperature, the beads were washed, pelleted by centrifugation and the Protein A-Sepharose-bound fraction eluted by boiling. The products were analysed by SDS-PAGE and fluorography. The lanes contain: A, total translation products. B-H, products bound by the different cDNA clones and immunoprecipitated by chitinase antiserum.

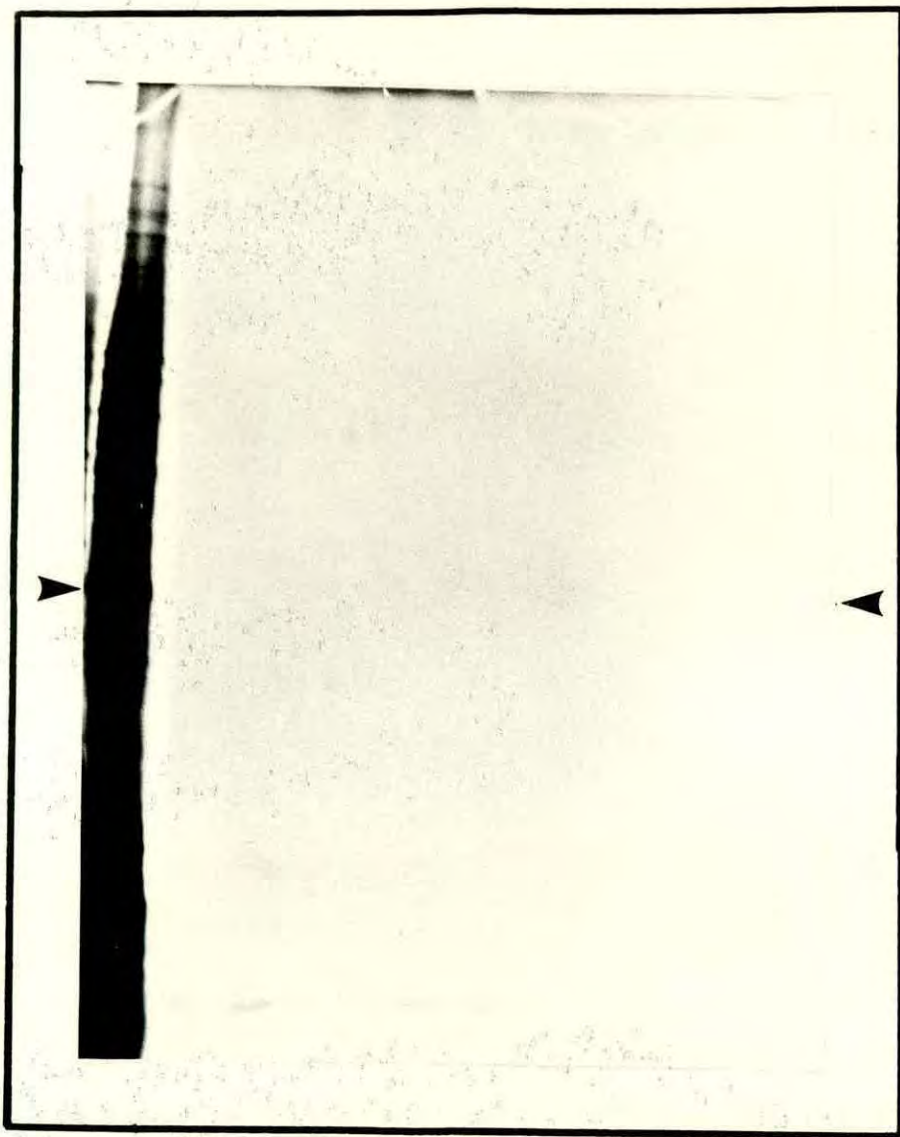


Figure 27 In vitro translation of hybrid selected mRNA's without immunoprecipitation

A repetition of the hybrid-release experiment illustrated in the figure 26 produced no visible bands when immunoprecipitation was not performed, even after long exposure of the gels. The lanes are the same as in the figure 26. The first lane contain total translation products. The fluorograph was overexposed to enhance any possible band. The lanes indicate the position of the 32,000 in vitro translation product.

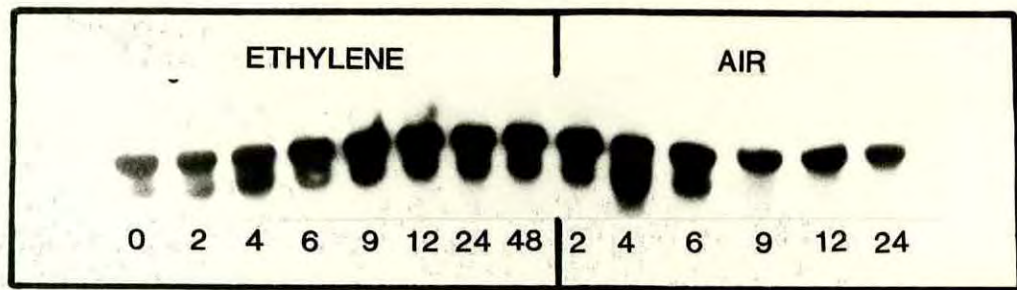


Figure 28 Northern blots hybridized with nick-translated pAK 11

Bean explants were incubated in ethylene or air for different periods of time. Abscission zones were excised and total RNA extracted from them. The RNA was loaded (20 ug per track) on a 1.2% agarose gel containing formaldehyde and electrophoresed at 10 v/cm in MOPS buffer pH 7. After electrophoresis, the RNA was blotted on to nitrocellulose and subsequently hybridized to the nick translated pAK 11. The filters were washed and exposed to preflashed X-ray film at -70°C . The numbers refer to the incubation time in ethylene or air in hours.

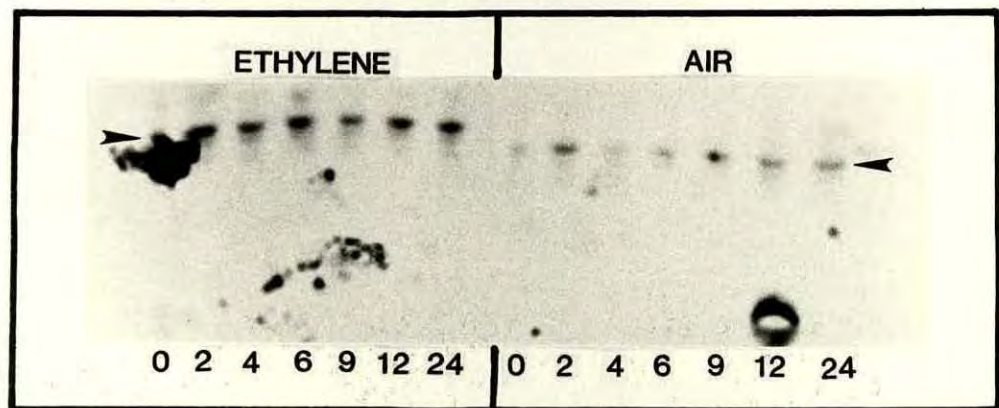


Figure 29 Northern blots hybridized with nick-translated pAK 3

The figure illustrates an identical experiment to that described in the figure 28, except that pAK 3 was used as a probe instead of pAK 11. The numbers refer to the time of incubation in ethylene or air in hours. The arrows indicate the position of the RNA species hybridizing to pAK 3.

present in RNA extracted from tissue kept in hypobaric conditions (figure 30), which does not contain chitinase sequences detectable by in vitro translation (figure 14). An experiment to hybridize both clones to the ribosomal clone pAT 71 produced negative results. None of the clones showed hybridization with pAT 71. Thus, the results obtained so far are ambiguous. I have identified by colony hybridization a group of clones containing an insert to sequences which appear to be present during ethylene induction. The clones were divided into two classes according to cross-hybridization. On hybrid release some of the clones seem to code for a polypeptide of 32kD which is immunoprecipitated by chitinase antibody. However, when I repeated the hybrid release without the immunoprecipitation no bands could be detected. In addition, the two classes of clones seem to hybridize to sequences present before and after ethylene induction but they do not hybridize to the ribosomal DNA clone (pAT 71). The identity of the clones, therefore, remains uncertain.

5.11 Discussion

The original aim in the construction of the two cDNA libraries was to isolate a chitinase clone. From the first library, one clone was identified (pABS 17) which seemed to select for a polypeptide of 32kD on hybrid release. This is the mw of the in vitro synthesized chitinase. The fact that pABS 17, strongly hybridized to

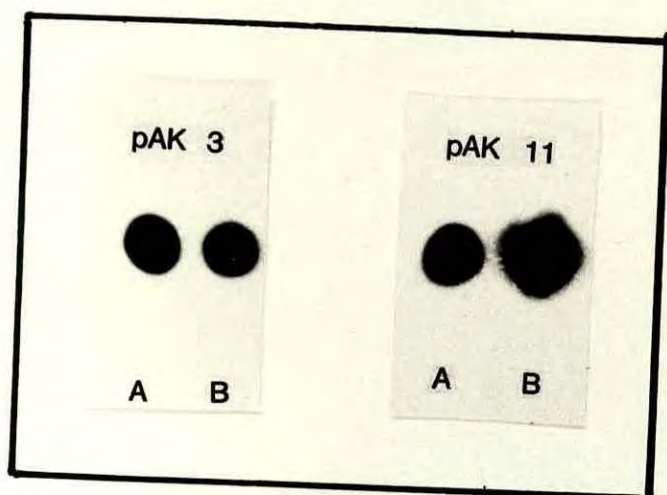


Figure 30 RNA dot blots hybridized to labelled DNA from pAK 3 and pAK 11

10 ug of total RNA was spotted on to nitrocellulose filters. The RNA was then hybridized to nick-translated pAK 3 and pAK 11. The filters were then washed and exposed to pre-flashed X-ray film at -70°C . The letters correspond to RNA from tissue kept in A, hypobaric conditions for 120 h. B, ethylene for 72 h.

a wheat rRNA clone (pAT 71) was an indication that there was some homology in sequence between them. These two results taken together suggested a mixed clone, containing sequences of both chitinase and ribosomal RNA. Even though the ribosomal DNA clone was from wheat, there have been reports that suggest sequence homology of rRNA genes between different plant species (Tanaka et al . 1980; Takaiwa et al . 1985). The possibility of a clone of this type is not unlikely. Clones containing mixed sequences have been identified before and characterised by sequence analysis and they have been attributed to rearrangements and duplications (Fagan et al . 1980). Reverse transcriptases can 'jump' from one substrate to the other thus producing spurious clones (Gilboa et al . 1979). On the other hand, clones to rRNA have been isolated following cDNA synthesis from poly A+ RNA in several other instances (Williams and Lloyd, 1980; Ohno et al . 1980; Sullivan et al . 1980; Thompson et al . 1980). In one case, when trying to obtain a cDNA clone to sucrose synthase in maize, about 25% of the total clones contained ribosomal sequences (Geiser et al . 1980). As can be seen this is not an uncommon problem. It has been suggested that preincubation of the cDNA preparation with excess rRNA before annealing with the vector, will eliminate sequences complementary to rRNA (Sullivan et al . 1980). This resultant mixed clone was unsuitable for our studies and consequently we undertook the

construction and screening of a second cDNA library.

From the second cDNA library 16 clones were identified by colony hybridization and hybrid release as containing a cDNA insert to products induced by ethylene. They seemed to code for a product which was immunoprecipitated by chitinase antibodies. This result was not reproducible when a hybrid release experiment without antibodies was performed on the clones. Thus, the results we had were ambiguous. So it was decided to investigate the homology of the clones with each other and use them in RNA blot experiments. The 16 clones were then cross hybridized and classified in two groups. A representative of each group (pAK 3 and pAK 11), which was the clone with the largest insert, was used in 'Northern' experiments with RNA from induced and non-induced plants. The clones strongly hybridized to sequences present in both types of RNA. If these results were genuine, assuming that the clones contained a chitinase sequence, they would argue for post-transcriptional control of chitinase by ethylene as the transcript would be present before and after ethylene induction. Ethylene would only 'activate' the message to make it translatable.

Chitinase has been shown to be induced by fungal elicitors and other stresses (Mauch et al. 1984; Metraux and Boller, 1985). Hypobaric treatment is an effective but drastic way to remove internal ethylene (Nilsen and Hodges, 1983). No chitinase message could be

detected by in vitro translation from tissue kept in these conditions. The profile of in vitro translation products is also different from that of ethylene treated tissue, suggesting that alterations have taken place in the pattern of protein synthesis. When RNA extracted from tissue kept in hypobaric conditions was probed with pAK 3 and pAK 11 a strong hybridization signal was obtained in both cases (figure 30). This result would seem to suggest, assuming that pAK 3 and pAK 11 contain chitinase sequences, that chitinase is still being synthesised in the absence of ethylene and under hypobaric conditions. Although such interpretation may be unlikely due to the detrimental effects of the treatment on the tissue, it cannot be ruled out. Perhaps the stress posed upon the plant by the treatment induces chitinase transcription but it cannot be detected by in vitro translation, The message can be detected by in vitro translation when ethylene is added back to the hypobaric chamber. As the identity of pAK 3 and pAK 11 is still unknown, my data is insufficient to establish whether chitinase is transcribed under hypobaric conditions. Cycloheximide has been shown to prevent chitinase induction by ethylene (Boller et al . 1983), which suggests that synthesis de novo , at least at the level of translation, is required for the induction. One possibility to explain the results obtained with the clones from the second library is that the result of the first hybrid release and immunoprecipitation experiment

is an artefact. The filters may not have been properly washed and so the wheat germ system was programmed not with one single mRNA species but with several. The antibodies may have precipitated, on translation, a product which bound unspecifically to the filters. This conclusion would be supported by the fact that without immunoprecipitation, I was not able to reproduce that result. More work is needed to determine the identity of these clones which is still ambiguous. However, they do seem to code for products responding to ethylene induction as indicated by the colony hybridization. Apart from the three main products which increase during ethylene treatment of abscission zones (figure 5), there may be other minor products that also accumulate. SDS-PAGE in one dimension may not be sensitive enough to detect them. Two dimensional gels would probably be the method of choice in this case. The clones might code for some of those products.

There are at least two approaches to trying to elucidate the identity of the clones:

- a) A primary sequence of the clones should help as it can be compared with the corresponding sequence deduced from the amino acid sequence of the protein.
- b) A heterologous hybridization using an identified clone of another organism.

Of this two possibilities only the second has been tried as the sequencing of the clones would be time consuming and I do not at present have the necessary expertise to

do it. A clone (pTOM 13) coding for an ethylene-regulated product was obtained (a kind gift from D. Grierson). The clone codes for a 35kD in vitro translation product which is induced by ethylene in ripening tomatoes (Slater et al . 1985). Wounding of the fruit can induce the synthesis of this product (D. Grierson, unpublished results). These observations were enough to suggest that pTOM 13 could be chitinase as wounding and ethylene can also induce the synthesis of chitinase (Boller et al . 1983; Metraux and Boller, 1985). On the other hand, the mw of in vitro chitinase (32 kD) was sufficiently close to that of the product coded for by pTOM 13 (35 kD). pAB 17, pAK 3 and pAK 11 were digested with Pst 1, run on an agarose gel and blotted on to nitrocellulose. The clones were then hybridized to the labelled insert of pTOM 13. None of the clones hybridized to the insert. This result was reproducible and suggested that there was no apparent homology between my clones and pTOM 13.

A different approach was then taken in the work, to try and obtain a clone for chitinase. A partial sequence of the purified enzyme was obtained and a mixture of oligonucleotides prepared, which was deduced from the amino acid sequence. The mixture was then used as a probe with a bean genomic library. This work is described in the next chapter.

CHAPTER 6 SCREENING OF A GENOMIC LIBRARY WITH AN OLIGONUCLEOTIDE MIXTURE

6.1 Rationale

The elucidation of partial sequences of proteins can be achieved by the Edman degradation method (Edman, 1950). From this information it is possible to deduce a partial nucleotide sequence for the corresponding mRNA. A mixture of oligonucleotides can then be synthesised, whose sequences represent all possible codon combinations predicted for a particular region in the protein. One of these sequences must be complementary to a region of the DNA coding for the protein. Mixtures of oligonucleotides are, therefore, potentially useful as hybridization probes for the screening of plasmid or bacteriophage libraries for particular sequences. This approach has been taken before in several instances to isolate a particular clone from genomic banks (Montgomery et al . 1978; Wallace et al . 1979; Szostak et al . 1979).

6.2 Aims

The aim of determining the partial sequence of chitinase and constructing the oligonucleotide mixture was to identify and characterize a genomic clone containing a chitinase sequence. This clone could then be used as hybridization probe to assess chitinase mRNA levels during ethylene induction and, in general, to study in more detail the regulation of chitinase by ethylene.

6.3 Characteristics of the Phaseolus vulgaris phage library

The library was obtained from Jerry L. Slighon (Agrigenetics). It was prepared by cloning 15-20 Kb fragments of Phaseolus vulgaris DNA digested with Mbo I into the lambda vector charon 34 (Loenen and Blattner, 1983). It had been screened (for phaseolin) and it was therefore an amplified library. The library was found to have a titer of 1,000,000 pfu/ml. Partial digestion of DNA with enzymes that recognize a 4 base pair sequence like Mbo I results in a random collection of DNA fragments (Dahl et al . 1981). This is an essential prerequisite if the bank is to have the entire genome represented (Dahl et al . 1981). Charon 34 can be used to clone DNA fragments up to 20 kb (Loenen and BLattner, 1983). It was hoped that the chitinase gene was represented in the library and was contained in one of these fragments.

6.4 Construction of an oligonucleotide probe to a partial sequence of chitinase

Using a protocol identical to that of Boller et al . (1983), chitinase was purified from abscission zones (section 4.2). A partial amino acid sequence of the N-terminus of pure chitinase was determined by L. Fothergill (Aberdeen University) by the Edman degradation method. This sequence has recently been independently determined by Lucas et al . (1985) and is identical to that reported here. This is not

surprising since the enzyme came from the same plant species in both cases. Based on our protein sequence, possible nucleotide sequences were deduced. The degeneracy of the genetic code results in ambiguity at the second base of some codons and at the third base of most codons. In order to minimize possible mismatches and based on the codon usage frequency in Phaseolus vulgaris (Hoffman et al . 1984) a short sequence of 6 aminoacids was chosen as a template for the oligonucleotides. Two mixtures of oligonucleotides corresponding to some sections of the enzyme were synthesised by S. Minter (Manchester).

Chitinase sequence determined:

| | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| glu | gln | cys | gly | arg | gln | ala | gly | gly | ala | leu | cys | pro |

| | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| gly | gly | asn | cys | cys | ser | gln | phe | gly | trp | cys | gly | cys | thr |

Oligonucleotide probe strategy

| | | | | | | | |
|-------------------------------|--|-----|-----|-----|-----|-----|-----|
| | | 4 | 5 | 6 | 7 | 8 | 9 |
| chitinase amino acid sequence | | gly | arg | gln | ala | gly | gly |

| | | | | | | | |
|---------------------|----|-----|-----|-----|-----|-----|-------|
| | | T | T | | T | T | T |
| | | C | C | G | G | C | C |
| coding sequence | 5' | GGG | CGG | CAA | GCA | GGG | GGG |
| | | A | A | | G | A | A |
| | | | | | | | |
| oligonucleotide 'a' | 5' | GGG | CGG | CAG | GCA | GGG | GGG |
| | | | A | A | T | | |
| | | | | | | | |
| oligonucleotide 'b' | 5' | GGA | CGG | CAA | GCG | GGA | GGT G |
| | | T | C | | C | T | A |
| | | | | | | | |

6.5 Screening the library with the oligonucleotides

The library was screened with the oligonucleotides which were end-labelled by kinase labeling with ³²P-ATP using

T4 polynucleotide kinase (Maniatis et al . 1983). By this method, a specific activity of 1.4×10^8 cpm/ug of DNA was routinely obtained. From 1.5 to 2×10^5 pfu were screened at a time. Plaque 'lifts' were made according to Benton and Davis (1977) in 9 cm petri dishes, in duplicate. The filters were initially hybridized to the probe for 16 h at 50°C in 6X SSC, 0.1 mg/ml heparin, 0.05% sodium pyrophosphate and washed in 4X SSC, 0.05% pyrophosphate at succesively 45°C for 20 min, 5°C for 15 min and 55°C for 45 min as described by Berchtold and Means (1985). The temperature of hybridization was calculated using the empirical formula for oligonucleotides of Suggs et al . (1981). Heparin was used as it is claimed to reduce the background more efficiently than the Denhardt's solution (Singh and Jones, 1984). On this first round of screening, the background was so high that potential positives were not identifiable (figure 31). As the filters were hybridized in duplicate, a true positive would line up on both filters. The 'background' consisted in a number of spots on the filters, which obscured true positives. The conditions were altered, therefore, and the filters were hybridized at a higher stringency: The temperature of hybridization was increased to 55°C and the salt concentration reduced to 3X SSC. However, the background was just as high as before.

It was then decided to substitute Denhardt's solution for heparin as I hypothesized that the heparin was not

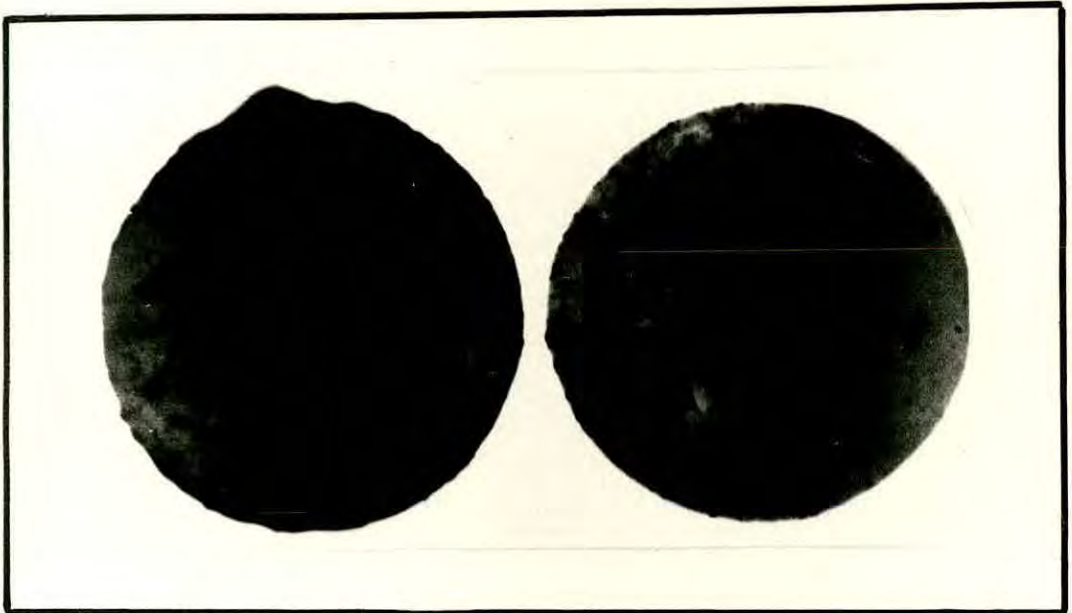


Figure 31 Hybridization of the oligonucleotide mixture to lambda genomic clones of bean

Lambda plaques were transferred on to nitrocellulose filters after being propagated in E. coli at a density of about 5000 plaques per petri dish. Each nitrocellulose filter was done in duplicate. The filters were then treated with denaturing and neutralizing solutions. After baking at 80°C for two hours, the filters were hybridized to the kinase labelled oligonucleotide mixtures. The filters were washed and exposed to pre-flashed X-ray film at -70°C. The figure illustrates two nitrocellulose filters replicates. The darkness of the autoradiograph is a reflection of the problems with the high background.

saturating the binding sites on the nitrocellulose. A new round of screening was performed in the same conditions as for the second screening except that 5X Denhardt's solution was used instead of heparin. In addition, for this third round of screening the washing conditions were modified to 50°C for 30 min and 55°C for 10 min in 3X SSC and 0.05% sodium pyrophosphate. This reduced the background considerably (figure 32) and 25 hybridizing plaques were obtained. These were picked, replated and screened as before. However they all turned out to be false positives. I was not able to get them to hybridize a second time even under different hybridization conditions.

A new round of screening was then performed at a lower stringency of hybridization (50°C and 4X SSC) in the presence of 5X Denhardt's and filters washed as in the third screening. It made no difference. No true positives were identified out of the 17 clones originally picked up.

A new approach was then taken. Wood et al . (1985) have published a method for using oligonucleotides as a probe which involves the use of the quaternary salt tetramethylammonium chloride (TAC). Tetraalkylammonium salts have been used to abolish the preferential melting of A:T versus G:C base pairs in fragments of DNA (Melchior-Von Hippel, 1973). TAC binds selectively to A:T base pairs (Shapiro et al . 1969) and in doing so, it displaces the dissociation equilibrium and raises the

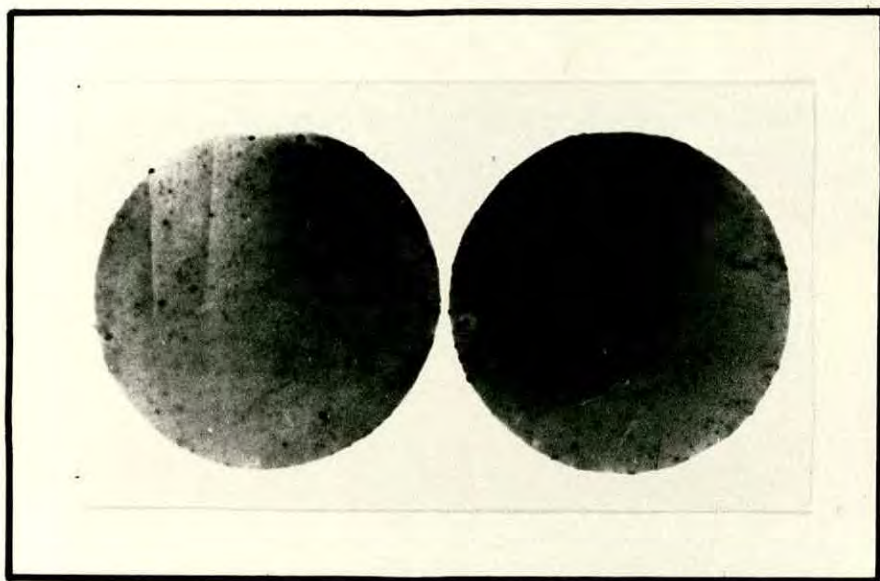


Figure 32 Hybridization of the oligonucleotide mixture to lambda genomic clones of bean under stringent conditions

This figure is a repeat of the experiment shown in the figure 31 except that the hybridization was performed under more stringent conditions. After hybridization, the filters were processed as described in the figure 31. The background was reduced considerably. Two nitrocellulose filters replicates are shown in this figure.

melting temperature. At 3 M TAC this displacement is sufficient to shift the melting temperature of A:T base pairs to that of G:C base pairs (Melchor-Von Hippel, 1973; Wood et al . 1985). So the stringency of the hybridization would only be dependant on the length and sequence similarity of the probe.

In this procedure, an initial non-stringent hybridization with the labelled probe is followed by washing with 3 M TAC to control the stringency of the hybridization. The hybridization was thus performed at 37°C in 5 X SSC, 50 mM sodium phosphate pH 6.8, 1% Denhardt's for 16 h. Filters were briefly rinsed with 3 M ~~TCA~~^{TAC} at 37°C and, before exposure to X-ray film, washed twice for 20 min in 3 M ~~TCA~~^{TAC} at 65°C. However, nothing was detectable on the filters. So the conditions were varied once again and the washes were performed at 50°C but the background was again too high. This was the last attempt. In conclusion, I was not able to identify a genomic clone containing a chitinase sequence from a lambda genomic library by using the oligonucleotides probe.

6.6 Discussion

Several attempts to isolate a genomic clone to chitinase were unsuccessful. The main problem with this type of experiments is the optimization of the signal to noise ratio. It is essential to enhance the signal at maximum while keeping the background to a minimum. To use synthetic oligonucleotides probes for hybridizations,

they must first be end-labelled and then annealed with single stranded DNA bound to a nitrocellulose filter. It is recommended that as a first approach the temperature of the hybridization be about 15°C below the estimated T_m of the hybrid (Szostak et al . 1979). The sensitivity of detection of the probe is determined by its length and the number of times its complementary sequence occurs in the DNA being probed, as well as for the stringency of the hybridization conditions. Also, if the conditions are not stringent enough, the probe may hybridize with many related but not identical sequences whereas the efficiency of the hybridization may decline with increasing stringency. A balance must therefore be found between the opposite requirements of sensitivity and specificity (Szostak et al . 1979).

Mismatches between the oligonucleotides and the filter-bound DNA are a problem in this type of experiments. It has been reported that even a single base pair mismatch in a 17-mer oligonucleotide has a significant destabilizing effect (Wallace et al . 1979). This problem is more critical when mismatches are in the middle of the sequence than at either end (Szostak et al . 1979).

In this study, a mixture of oligonucleotides was used, which contained many possible codon combinations for the sequence of interest. One of them should have been exactly complementary to the DNA coding for chitinase. This sequence would therefore have been expected to

hybridize to its corresponding sequence. However, I was not able to discriminate between true and false positives due to the problems with the background mentioned in previous sections. One possible and simple explanation might be that the radioactive probe stuck to bacterial debris due to improper washing of the filters. Another explanation might be that the hybridization signal was too weak and I took it for a false positive. The use of TAC, which makes the stringency of the hybridization to be controlled only by the length of the probe (Wood et al . 1985), did not prove useful in my case. The reasons for this are unclear.

The DNA bound to the filters comprised a whole population of sequences. During hybridization, the different oligonucleotides would be competing for their complementary sequence. Assuming an equal proportion of each oligonucleotide in the mixture, every oligonucleotide molecule would have the same probability of finding their counterpart. Oligonucleotides having incomplete homology may have the same or a similar affinity for their complementary sequence as oligonucleotides with complete homology. Consequently they may be able to initiate the formation of hybrids in certain conditions. However they may not be able to complete the formation of hybrids because of the stringency of the hybridization or the washes. In the end the 'wrong' oligonucleotides would be preventing potential positives from initiating a hybrid and from

being detected.

Montgomery et al (1978) reported the use of an oligonucleotide as a probe to isolate the yeast cytochrome C gene. While the screening of the library was eventually successful, the authors pointed out the many difficulties which were encountered. Among them, were the poor reproducibility of hybridization of the ³²P-labelled probe from experiment to experiment and the weak signal obtained with the labelled probe during the screening of the plaques.

Lamba clones suffer the disadvantage of potential deletion of duplicate segments of DNA during propagation in E. coli (Loenen and Blattner, 1983). This is particularly so for plant DNA which contains a high proportion of repeated sequences (Goldberg, 1983; Flavell, 1980). On the other hand, amplification of libraries might lead to selective loss of specific sequences (Dahl et al . 1981). Thus it might have been that the bean library, which had been amplified and screened once did not contain a chitinase sequence or it was underrepresented for the reasons mentioned above.

Clearly, a major limitation in this experiments was the lack of a suitable gene probe, like an homologous gene, for the sequence of interest. Several factors like the ones discussed above may have contributed to this lack of success but the true reasons are uncertain.

CHAPTER 7 GENERAL DISCUSSION

7.1 Aims

The intention of this chapter is to summarize the major results presented in preceding chapters and to discuss their relevance in the wider context of higher plant development. The validity of the main techniques employed will be evaluated and possible improvements suggested. Future area for research will also be discussed.

7.2 Summary of results

The principal achievements of the research presented in this thesis are outlined below:

1) Chitinase has been purified from abscission zones. A radiometric assay was developed and used to show that chitinase is present in an active form and that it can be induced by ethylene. Removal of ethylene prevented chitinase induction.

2) Chitinase mRNA was identified by virtue of its in vitro translation product in a wheat germ cell-free system. Several lines of evidence were used to reach this conclusion. It was found that in vitro chitinase has a mw 2 kD higher than the mature enzyme. Microsomal membranes can process the 32 kD product to one of 30 kD. This suggests that, if there is a signal peptide present in the enzyme, chitinase may be an enzyme transported across membranes.

3) Antibodies were raised against purified abscission zone chitinase and used in various immunoprecipitation

experiments.

4) By enzymatic assay and detection by antibodies, chitinase was found to be present in stems, petioles and leaves after ethylene treatment.

5) The time course of induction of chitinase by ethylene is similar in every organ tested, but it reaches lower specific activities in stem. These results are supported by the in vitro translation results in which stem showed the lowest levels of translatable chitinase mRNA.

6) The application of auxin did not have an effect on chitinase induction by ethylene. As auxins are strong inhibitors of abscission, this observation suggests that chitinase does not play a direct role in abscission. Chitinase mRNA was still detectable when abscission was inhibited by auxins.

7) Two cDNA libraries were prepared and screened. A group of clones was selected as containing inserts coding for ethylene-regulated products. However, the identity of the clones is still unknown.

8) A partial amino acid sequence of chitinase was obtained and, deducing the necessary information from it, an oligonucleotide mixture prepared and used to screen a bean genomic library. However no clones containing chitinase sequences could be identified.

7.3 Discussion

The general aims of this study were to contribute to the existing knowledge on the regulation of gene expression by PGS and the process of abscission. I used explants of

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bean seedlings as experimental system. The particular purpose of this work was to establish an identity between the 32 kD in vitro translation product that accumulates during abscission and chitinase, an ethylene-regulated enzyme present in bean leaves. In discussing my results I intend to correlate the changes in the parameters outlined above. Most of the main results have already been analysed in the relevant chapters, consequently the discussion in this chapter will be of a general nature.

How valid is the experimental system used in this study?

The process of abscission is a very important developmental stage in the normal life cycle of a plant. Abscission is frequently utilized by the plant for the purposes of homeostasis as in the removal of senescent, injured or infected organs as well as for the recycling of nutrients. The process is sensitive to a number of internal and external conditions (Addicott, 1982; Sexton and Roberts, 1982). Among them, PGS play an important role (Addicott, 1970).

The development of the explants method by Addicott and co-workers (Addicott et al . 1949) was a significant advance in methodology. There have been a number of papers on abscission, all of which have employed this technique. The most obvious disadvantage is the starvation effects as the excised pieces of tissue are left for up to three days without an external supply of

nutrients, salts and water, although they are kept in a humid atmosphere. All of this may have detrimental effects on the system (Jacobs, 1962).

Exposure of the tissue to possible invasion by microorganisms and induction of wound ethylene are possible consequences of the excision of the explants from the intact plant. On the other hand even though the explants can be made to abscise in reasonable synchrony, the leaves in the intact plant may not abscise at a comparative physiological state in natural conditions. Clearly, the nutritional status of the explants and the intact plant will be different.

Ethylene can induce abscission in explants and for that reason they have been used in studies on the natural regulation of abscission. The minimum ethylene concentration needed to cause abscission in bean explants is 0.1 ppm and the saturating concentration 10 ppm (Abeles and Ganahan, 1968). Our system can be criticized on the ground that we used an ethylene concentration five times higher than the saturating concentration. This was in order to make the explants abscise in synchrony. The possibility that this high concentration of ethylene may have detrimental effects on the system cannot be ruled out. However, in spite of all this, it has been claimed that no major differences in the biology of the explants and the intact plant have emerged, since this methodology was introduced (Sexton and Roberts, 1982).

Cell separation during abscission does not occur throughout the entire abscission zone but is limited to a narrow layer, 1-3 cell wide (Gawady and Avery, 1950; Halliday and Wangermann, 1972). It can be appreciated that enough experimental material is not easily obtained. For most experiments, a small section (3 x 5 mm) of the explants, containing the abscission zone (figure 1), was employed. Undoubtedly, this small piece of tissue will have contained some contaminant adjacent tissue. No attempts were made to investigate differences in protein composition between adjacent sections of the abscission zone. However, when in vitro translation products from petiole, stem and abscission zones were compared, some differences in the profile of translation products were obvious (figure 3). This was particularly so in the case of the stem. Petioles and zones showed somewhat similar profiles of translation products, the main differences being of a quantitative nature. This indicated that the sections of tissue used as abscission zones were sufficiently different and that contamination by adjacent tissue was apparently not a major problem.

What is the nature of the molecular changes during abscission?

There are a number of enzymes which become conspicuous during abscission together with an increased metabolic activity and an increase in the synthesis of RNA (see section 1.5.3). Work with inhibitors suggested that de novo synthesis of proteins was involved in the process

(Abeles and Holm, 1967; Holm and Abeles, 1967). Lewis and Varner (1970) showed cellulase, an enzyme believed to be involved in abscission, to be synthesised de novo during abscission. Several species of mRNA, whose in vitro translation products have mw of 42 kD, 32 kD and 17 kD, accumulate during abscission (figure 3). These products may be involved in abscission. As a result of the work by Boller et al . (1983) I hypothesized that one of those species of mRNA (32 kD) was chitinase. My work from then on was to try to establish this identity.

Is the 32 kD in vitro translation product chitinase?

Using several lines of evidence, the 32 kD in vitro translation product which accumulated in abscission zones was identified as chitinase (See full discussion in chapter 3). By using a specific assay for chitinase it was first established that chitinase was present in abscission zones and that it was detectable by virtue of its activity. The enzyme was induced by ethylene and started accumulating after a lag of 6 h. Chitinase was purified to homogeneity from abscission zones following a protocol similar to that of Boller et al . (1983). The purified enzyme was used to prepare antibodies. The antibodies were shown to be effective as they abolished chitinase activity. These antibodies were used in different immunoprecipitation experiments.

The wheat germ system programmed with total RNA extracted from ethylene-treated abscission zones, was challenged with chitinase antibodies. The antibodies

were able to precipitate the 32 kD in vitro translation product. This result was consistently obtained. The experiment was repeated using in vitro translation mixture programmed with total RNA extracted from petiole and stem and in every case the 32 kD translation product was precipitated by the chitinase antibodies. The antibodies were also used to precipitate chitinase from tissue labelled in vivo with S-methionine. In several immunoprecipitation experiments in vitro a contaminant band could be detected. This band had a mw of 42 kD. This contaminant may be the same as that obtained as a minor band in immunoelectrophoresis experiments. The reasons for this contamination are uncertain. The preparation of chitinase used for raising the antibodies was pure as judged by SDS-PAGE. It is possible that some minor component was present in the preparation and that SDS-PAGE was not sensitive enough to detect it. Antibodies would have then been produced against this component. However, it is also possible that the antibodies recognised a polypeptide having some antigenic determinants in common with chitinase. There are indications that the 42 kD in vitro translation product may be cellulase (Kelly et al . in preparation). The substrates for chitinase and celulase have a rather similar structure. They share linkage similarity and monomer sugar similarity, but with the extra N-acetyl group in the chitin monomer. There may be,

therefore, some structural similarity in the active site of chitinase and cellulase which could be a reflection of the apparent similarity in substrates. In this respect, it is interesting that a 32 kD band was obtained as a contaminant in experiments to immunoprecipitate avocado cellulase from in vitro translation mixtures (Tucker and Laties, 1984; Christoffersen et al . 1985). Thus, cellulase and chitinase may share antigenic determinants.

Mature chitinase has a mw of 30 kD (Abeles et al . 1971; Boller et al . 1983). The 2 kD discrepancy with the 32 kD product could be due to the presence of the signal peptide on the in vitro translation product. It has been suggested that secreted proteins usually contain a signal sequence in order to be secreted (Blobel and Dobberstein, 1975; Walter and Blobel, 1985). The signal is clipped off during 'processing' of the protein. Addition of bean microsomal membranes results in the processing of the 32 kD in vitro translation product to one of 30 kD which is the mw of mature chitinase. This observation suggested that chitinase may be a secreted enzyme. This has turned out to be the case with yeast, in which chitinase was found to be located in about equal proportions in the vacuole and the extracellular space (Elango et al . 1982). In experiments with yeast protoplasts chitinase was shown to be initially located in the vacuole and later secreted (Elango et al . 1982). Bean chitinase has

been shown to be similarly present in the vacuole (Boller and Vogelli, 1984), which compares favourably to yeast chitinase. It is possible that bean chitinase may be an enzyme which is processed, sequestered into a cellular compartment and subsequently secreted.

Secretion of enzymes in plants has been proposed to follow a vesicular route. After synthesis, the enzymes would be transported into the lumen of the endoplasmic reticulum and subsequently encapsulated in a vesicle for movement through the cytoplasm to the plasmalemma (Laidman, 1980). This has proved to be the case for barley alpha-amylase and protease (Gibson and Paleg, 1975, 1976; Firn, 1975). In this respect, ethylene has been implicated in the secretion of peroxidase (Ridge and Osborne, 1970), alpha-amylase (Eastwell and Spencer, 1982) and cellulase (Abeles and Leather, 1971). In the case of cellulase, ethylene can also induce synthesis (Lewis and Varner, 1970). It is possible that chitinase, whose synthesis is induced by ethylene, follows a similar route, if secreted. Ethylene might also regulate its secretion.

The purification of chitinase involves an affinity chromatography step (Boller et al. 1983). This is a rather specific purification method as only molecules with the same or a similar affinity for the substrate (chitin) will bind to the column. The in vitro translation mixture was run through this affinity column, the column was washed and the bound material

eluted. The products were analysed by SDS-PAGE. The most prominent band that could be detected on the gel after all this procedure was the 32 kD product, which suggested that the 32 kD in vitro translation product had apparently a similar affinity for chitin. The 42 kD product, a strong candidate to be cellulase, could sometimes be observed on the gels, which suggests again a possible relation between cellulase and chitinase.

Attempts to confirm the identification of the 32 kD product as chitinase by partial proteolytic digestion were unsuccessful. No clear-cut results were obtained by this method. However, the evidence provided by other methods strongly suggests a common identity. The final confirmation will only be obtained by comparing the primary sequence of both products.

How is the chitinase induction by ethylene manifested?

From the data presented in this thesis it is clear that chitinase is present in abscission zones in addition to being in petioles, stems and leaves. The enzyme is present in an active form as suggested by the fact that chitinase activity can be detected. This assumption is made bearing in mind that the situation in the intact plant may be different from that in vitro. In vitro assays do not necessarily reflect the activity of an enzyme in natural conditions. The enzyme might be present in an inactive form and the extraction might somehow activate it; for instance by bringing it into contact with factors which would otherwise not be

available to the enzyme. On the other hand, one can never be certain that an extracted enzyme is fully active. Essential cofactors might be lost during extraction and thus the activity in vitro would be lower than it should be.

Chitinase was purified from abscission zones following a protocol similar to that of Boller et al . (1983). By this procedure the enzyme was purified about 17-fold. In contrast, Boller and co-workers obtained a 24 fold purification. The reasons for this difference is unknown as the protocol was the same in both cases. Inactivation of some enzyme during extraction might be an explanation. Even though there was some chitinase activity detectable at the beginning of the ethylene treatment, the activity did not start accumulating significantly until after 6 h of the treatment. This was in agreement with previous observations in bean leaves (Boller et al . 1983). The increase in activity followed similar kinetics in every organ tested, although it reached lower specific activities in stems.

A plot of the product formed as a function of added enzyme did not yield a straight line. The reasons for this are unclear. Only initial velocities were proportional to the amount of enzyme added and consequently they were used to calculate enzyme units. The substrate for the assay was regenerated chitin, prepared by reacetylating chitosan (Molano et al . 1977). Decomposition of only 1-2% of the substrate was

enough to give accurate measurements (Molano et al ., 1977). Chitin usually forms a part of a very complex system, in association with proteins and other carbohydrates, and it is not found alone (Foster and Weber, 1960). The chitin chains are also tightly hydrogen bonded together. Chitin may therefore be only partially accesible to the enzyme. The lack of linearity of the reaction of chitinase with its substrate has been interpreted as a possible time-dependent change in the substrate. Addition of fresh enzyme to the reaction mixture increased the rate only slightly suggesting that a modification had taken place in the substrate (Molano et al . 1977).

The increase in enzymatic activity seems to be correlated by an increase in chitinase antigen as shown by the 'western' experiments (section 4.5.2). Crude homogenates were made of tissue at different stages of the ethylene treatment. The homogenates were run on SDS-PAGE, transferred to nylon membranes and challenged with chitinase antiserum. The antibodies were detected with radioactive protein A. The chitinase band did not appear until after 6 h of the ethylene treatment and it accumulated thereafter. These results are in agreement with those of the enzymatic assays (section 4.5.1) where the chitinase mRNA did not appear until 6 h of the treatment. These results taken together suggests that the increase in enzymatic activity is correlated with an increase in chitinase protein.

The induction of chitinase by ethylene at the mRNA level

The properties and function of mRNA molecules cannot be assayed by simple radioactive amino acids incorporation measurements but only by the analysis of the radioactive products synthesised. This is generally accomplished by SDS-PAGE in one or two dimensions which can resolve polypeptides from complex mixtures. Cell-free systems have been employed to assay particular mRNA species and their products.

The cell-free system employed in this study to analyse mRNA translation products was the wheat germ (Roberts and Patterson, 1978). Bottomley et al . (1976) have shown that the wheat germ system will primarily translate cytoplasmic mRNA, observations confirmed by Walden and Leaver (1981) who also showed that cell free systems can be used to study developmentally regulated changes in the amount of mRNA products.

The wheat germ system, however useful, has several disadvantages. Firstly, it is known that different mRNA molecules may have different efficiencies of translation (Lodish, 1974). Therefore, the complexity of in vitro translation products obtained programming the wheat germ system with a particular mRNA preparation need not necessarily reflect the complexity of input mRNA. Secondly, with this system, only accumulation of translatable mRNA can be detected. Therefore, in vitro translation cannot distinguish between transcriptional

control, changes in translatability of mRNA or control of mRNA degradation. Lastly, the efficiency of any in vitro translation system is much lower than that in vivo. It is reckoned that at least in E. coli lysates the rate of elongation in vitro is less than 0.05 peptide bonds per second per ribosome while the corresponding rate in vivo is 200 to 400 times faster, as roughly only one tenth of the ribosomes are active in vitro (Kurkland, 1982). However, in spite of this, the utility of the in vitro translation systems is clear.

The analysis of the in vitro translation products of RNA extracted from abscission zones reveals a complex profile of products (figure 5). This was also the case with products of RNA from stem, petioles and leaves (figures 3 and 4). The presence of high mw products suggested that the RNA preparation was not extensively degraded during isolation. Three in vitro translation products (42 kD, 32 kD and 17 kD) accumulate during abscission and their synthesis appears to be dependant on the presence of ethylene. There are suggestions for the 42 kD product to be cellulase (Kelly et al. in preparation), an enzyme believed to be involved in abscission (Horton and Osborne, 1967; Sexton et al. 1980). The 32 kD product has been tentatively identified as chitinase but direct comparison of the primary sequence of both products is needed to confirm this identity. There is still no identity assigned to the 17

kD product.

No abscission zone-specific mRNA's were identified by this method of analysis. This was unexpected, since Osborne et al .(1985) used immunological methods to show that during abscission, unique proteins appear in the abscission zone of Sambucus . Lack of resolution of the methods employed here may explain the failure to find specific abscission zone mRNA's. It is possible that there was some tissue specificity since the 42 kD, 32 kD and the 17 kD products were the major species in the petiole and the abscission zone. In stems and leaves, these products were not as abundant. However, this 'specificity' may only be a reflection of the fact that in this study the in vitro traslations were programed with standardised amounts of total RNA. Therefore, no account was taken of the actual concentration of mRNA in a particular tissue nor the traslational efficiency, which may vary between different mRNA's (Lodish, 1974). Measured rates of protein synthesis indicate that at the time of separation, the rates in the zone are far faster than those in the distal tissues (Osborne, 1968). Therefore, a particular mRNA species might be synthesised both in the abscission zone as in adjacent tissues but translated at a greater rate in the zone. A densitometric analysis (figure 15) of the translation products of figure 5 reveals that chitinase accumulates substancially within 6 h of ethylene treatment. It apparently reaches a peak by 12 h. In the absence of

data on chitinase accumulation between 0 and 6 h of ethylene treatment, it is difficult to correlate the increase in chitinase activity and protein with the increase in chitinase mRNA. The accumulation of chitinase mRNA within 6 h of the treatment may follow a different kinetics from that shown in figure 15, which is only an extrapolation.

The profile of in vitro translation products of zones and petioles is rather similar, with the three major products accumulating. The profile of translation products of leaves and stems was different from that of zones and petioles. In stems the 17 kD product was the only product which seemed to accumulate substantially, although there were other polypeptides which showed an increase or a decrease on ethylene induction. In leaves, the most abundant product was a 20 kD product which was detectable all the time either in air or in ethylene. The small subunit of Ribulose biphosphate carboxylase (Rubisco) has been reported to be coded by the nucleus and to be synthesised as a higher mw precursor of about 20 kD in pea (Highfield and Ellis, 1978). It is interesting to speculate that the 20 kD band found in my gels may be the precursor for the small subunit of Rubisco.

The profile of translation products obtained with RNA from leaves looks somewhat similar to that obtained by Giles et al . (1977). They analysed in vitro translation products of RNA from Phaseolus vulgaris

leaves grown under different light regimes and concluded that there were several changes in the translatable mRNA population induced by far red light. They interpreted this observation as mobilization of mRNA from a stored form. It is interesting to note that one of the major polypeptides which Giles et al . (1977) reported as being induced by light should have been one of 32 kD. That polypeptide may be the same as the 32 kD band on my gels. If that is the case, the meaning of their finding is unknown, in the light of this research.

In a broad sense the profiles of products of RNA extracted from leaves treated with ethylene or with air look rather similar (figure 4). However several changes can be detected. The 42 kD and the 32 kD bands can be seen only to accumulate in leaves treated with ethylene. The 17 kD product was not detectable in leaves. There is a group of proteins of mw about 30 kD which was detectable regardless of the presence of ethylene. These proteins could be seen to decline after 9 h. The meaning of this and the identity of these polypeptides are uncertain. The major complexity of the in vitro translation product of leaves in comparison to those of abscission zones is clear. Perhaps for that reason, chitinase does not make up the same relative proportion in abundance in leaves as it does in zones.

A peculiar observation made during all these experiments is that the relative proportion of chitinase in the population of translatable mRNA was variable depending

on the batch of tissue and the season of the year. I found that some batches of tissue had abnormally low levels of chitinase as judged by in vitro translation. The same was true for tissue grown in a particular season (winter in this case). In one set of experiments using a variety of beans other than Red Kidney, the chitinase content was very low. Chitinase levels can apparently vary depending on, amongst other factors, the variety of beans (F.B. Abeles, pers. commun.).

Attempts to obtain a clone to chitinase

Several attempts to identify a clone to chitinase were unsuccessful. Two cDNA libraries were constructed and screened but no chitinase sequence could be identified. One clone was identified from the first library by hybrid release as containing a chitinase sequence (pABS 17). However, it also seemed to contain rRNA sequences as indicated by the strong hybridisation to pAT 71, a wheat rRNA clone. This result made it unsuitable for our purposes. Obtaining clones containing rRNA sequences is not an uncommon event as this has occurred in several instances before (Sullivan et al . 1980; Ohno et al . 1980; Geiser et al . 1980; Williams and Lloyd, 1979; Thompson et al . 1983). Reverse transcriptase can apparently use rRNA as substrate in the absence of primer (Sullivan et al . 1980). It can also 'jump' from substrate to substrate (Gilboa et al . 1979). These two observations might explain why pABS 17 contains a mixed sequence. Alternatively, there could be

a common sequence between chitinase and rRNA.

The screening of the second cDNA library produced 16 clones which seemed to code for products induced by ethylene. 12 out of 16 clones appeared to contain chitinase sequences as suggested by hybrid release and immunoprecipitation by chitinase antibodies. However, hybrid release without immunoprecipitation did not confirm this result. No translation products could be detected, even after long exposure of the gels. The clones were cross-hybridized and subdivided into two groups. A representative of each group was then used with RNA blot experiments. They hybridized to sequences present in the absence or presence of ethylene, although the hybridization seemed to be stronger in the case of sequences present in the presence of ethylene. They also showed hybridization with RNA extracted from tissue kept in hypobaric conditions which was deficient in chitinase sequences as suggested by in vitro translation. They did not show hybridization to the rRNA clone. All these results did not allow me to give a definite answer about the identity of the clones. Their identity, therefore, remains unknown. They might code for in vitro translation products which are present all the time regardless of the presence of ethylene. As judged by in vitro translation results there are several products which fall into this category.

One of the main criteria used to establish the identity of a clone is the hybrid release method (Ricciardi et

al . 1979). This is sometimes complemented by the 'opposite' method, hybrid arrest translation (Patterson et al . 1977) in which one assays by in vitro translation for the disappearance of a product which is selected for by a corresponding clone. In this study, I found that the hybrid release method gave variable results. On occasions the results were not reproducible. There must be several factors affecting the final outcome of the experiment. A critical factor may be the amount of poly A+ RNA used for the hybridization. It is reckoned that amounts in excess of 25 ug of poly A+ RNA per filter give a good and reproducible hybridization (W. Schuch, pers. commun.). A major limitation in my case was the amount of poly A+ RNA obtainable from abscission zones. A typical RNA preparation yielded about 70 ug of total RNA per gram of fresh tissue. Approximately 1% of this RNA was poly A+. About 50 abscission zones are needed to make up a gram which will produce 0.7 ug of poly A+ RNA. To obtain 25 ug of poly A+, 1800 abscission zones are required. We could only grow 40 seedlings in each tray, which represented 80 abscission zones. We would need 22 trays of bean seedlings to recover enough poly A+ RNA for hybridization with one clone. Clearly, enough poly A+ RNA to screen all the clones I had, was not easily obtained. This was the reason why total RNA (400 ug) was employed for the hybrid release experiments. However, the concentration of poly A+ RNA present per clone may

have been still very low (about 4 ug), and might explain the variability found with this method.

It is possible that the result shown in figure 26 is an artefact caused by insufficient washing of the filters. The cell-free system might have been programmed not with the mRNA selected by the clones but several other mRNA species may have been present. One of the 'contaminant' mRNA species could have been recognised, on translation, by the antibodies. More work is needed to establish the identity of these clones. Also, attempts to identify a clone containing chitinase sequences from a bean genomic library were unsuccessful. Several possible explanations are discussed in the relevant chapter.

Clearly a major limitation in these experiments was the lack of a suitable and defined gene probe. I did not find the oligonucleotide mixture satisfactory as probe.

A clone coding for an ethylene-regulated product from tomato (pTOM 13, a kind gift from D. Grierson) was hybridized to pABS 17, pAK 3 and pAK 11. This clone looks like a good candidate to be chitinase (Slater et al . 1985). However, none of my clones hybridized to pTOM 13. This result may be explained in several ways; if pTOM 13 is chitinase, then none of my clones contains a chitinase sequence recognized by it. On the other hand, tomato chitinase may be different in sequence from bean chitinase and, consequently, they would not cross-hybridize. A final possibility is that pTOM 13 is not chitinase. In conclusion as no

chitinase clone was identified, no correlation could be made between the data obtained from other studies and chitinase transcripts.

Certainly, the best approach to confirming the identity of a particular clone is to obtain the primary sequence and compare it with that of the protein. One could potentially identify the coding sequence as well as possible regulatory sequences.

Is chitinase involved in abscission?

During abscission, there is evidence of cell wall degradation (Sexton, 1976; 1979). Chitinase, as a hydrolytic enzyme, might have a role in the process. However, to my knowledge no chitinase substrate has been found as yet in plants. Attempts to detect one have been unsuccessful (Boller et al . 1983). This does not exclude the possibility for chitinase to have a still undetected substrate in abscission zones. Alternatively, chitinase may protect the plant against infection of the scar left by the fall of the leaf (figure 33). The exposed surface may be particularly susceptible to infection.

Ethylene is a strong promoter of abscission (Jackson and Osborne, 1970; Sexton et al . 1985) and auxins can delay the process (Horton and Osborne, 1967; Sexton et al . 1980). Chitinase is only sensitive to ethylene as indicated by the increase in mRNA (figure 5), enzyme protein (figure 20) and enzymatic activity (figure 19). Auxins had no effect on the accumulation of chitinase

Figure 33 Scanning electron micrograph of the exposed surface of a bean abscission zone after abscission

The fall of the leaf leaves a scar which may be susceptible to infection by pathogens. Chitinase may be present in abscission zones to protect them against invasion by pathogens (figure kindly provided by Roy Sexton).



mRNA as detected by in vitro translation when applied in conditions which prevent abscission (Sexton et al . 1980). This observation and the fact that chitinase is found all over the plant suggest that chitinase does not have a specific role in abscission. No attempts were made to assay for chitinase activity in tissue treated with auxins.

What is the function of chitinase in the intact plant?

The evidence quoted in the section 1.6.3.4 strongly suggests an involvement of chitinase in reactions of defense against pathogens. Chitinase has been shown to inhibit the growth of fungi in tomato leaves (Pegg and Vessey, 1973; Pegg, 1976). Chitinase can be induced by fungal elicitors (Mauch et al . 1984; Metraux and Boller, 1985). The elicitors may function as messengers by altering the metabolism of receptive plant cells so that the plant can respond to an invasion by pathogens by producing compounds able to stop or prevent the development of disease (section 1.6.2.4).

Synthesis of stress ethylene is generally a consequence of, amongst other things, wounding and fungal invasion (Yang, 1980; Abeles, 1985). As chitinase can be induced by ethylene (Abeles et al . 1971; Boller et al . 1983; this thesis), chitinase may also be induced by stress ethylene. However, the evidence seems to indicate that stress ethylene is only a symptom but not a necessary signal for the induction (Boller et al . 1983; Mauch et al . 1984).

7.4 FUTURE DIRECTIONS

The research described in this thesis was part of a larger programme aimed at increasing the understanding of the molecular mechanisms regulating abscission in beans. The analysis and characterization of the different enzymes involved in the process is an essential first step towards this goal.

The limitations of the techniques employed, outlined in previous sections, to determine the level of control of gene expression are clear. Recombinant DNA methodology can provide useful gene probes which can then be used to study the process of abscission further. This is also the case when studying the effects of PGS on gene expression. The effects of several stimuli on abscission can be evaluated in the light of this methodology which, when combined with information obtained from other studies, can provide more definite answers.

Work is in progress to try to obtain a clone for the 42 kD product, a good candidate to be cellulase, and for chitinase. Antibodies are available for both enzymes. The combination of gene probes and antibodies will allow us to obtain further insight into the process of abscission and confirm whether chitinase has a role in it.

The analysis of other enzymes which are believed to be involved in abscission, such as polygalacturonase, should help to increase our understanding of the process. Eventually it should prove possible to

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correlate molecular, biochemical and ultrastructural evidence to provide a complete picture of abscission.

A long term goal is to understand abscission from a point of view of the intact developing plant. Abscission along with most other plant developmental processes has been considered a process of correlation of many factors (Addicott, 1982). The numbers of factors involved may be extensive. The availability of cloned gene probes along with other experimental tools should increase the understanding of this complex and fascinating process.

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